EVALUATION OF NATIVE ISOLATES OF *Bacillus thuringiensis* AND *Metarhizium anisopliae* FOR THE CONTROL OF *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)

By

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B.Ed. (Science) (Hons)

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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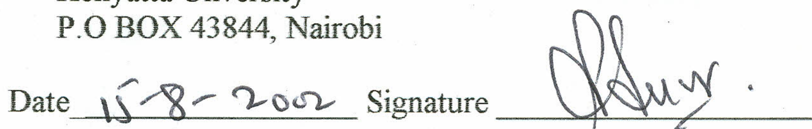

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DEDICATION

To my parents Raphael and Jecinta, Brothers and sisters and my fiancée Gladys for their love and support.

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ABSTRACT

Twelve isolates of *B. thuringiensis* and three of *M. anisopliae* were isolated from the soil and cadavers obtained from diverse geographical regions of Kenya. Pathogenicity of fifteen isolates of *B.thuringiensis* and fourteen of *M. anisopliae* to *P. xylostella* was determined in the laboratory. Mortality caused by *B. thuringiensis* to the first instar larvae varied between 6 and 100%. Five isolates caused mortality of 100 % in the first day after inoculation. Lethal time for the most virulent isolate was 0.5 days and 8.3 days for the less virulent. There were also differences in the pathogenicity of isolates of *M. anisopliae* against *P. xylostella* larvae. Mortality ranged from 40 -100 %. Two isolates, ICIPE 20 and KZ-4 were the most pathogenic causing 100 % within 5 days. Isolate ICIPE 30 was the less pathogenic as it caused mortality of 40 % in 8 days. Isolate ICIPE 20 had the shortest LT_{50} of 2.3 days and ICIPE 30 the longest of 8.1 days.

Dose - mortality response against *P. xylostella* larvae was determined for four isolates of *B. thuringiensis* (KF-2, MR-1, NA-2, NA-3 and Px-K3) and two of *M. anisopliae* (ICIPE 20 and KZ-4). Mortality was dose dependent for the two entomopathogens. Lethal dose for the most pathogenic *B. thuringiensis* isolate was 1.2 x 10^5 and 5.1 x 10^5 spores ml^{-1} for the less pathogenic isolate. Lethal doses for *M. anisopliae* isolates ICIPE 20 and KZ-4 were 2.3 x 10^5 conidia ml^{-1} and 3.9 x10^6 conidia ml^{-1}, respectively. Different larvae stages of *P. xylostella* exposed to both entomopathogens varied in their susceptibility to infection. Younger instars were more susceptible than the older larval instars. Lethal dose for the most susceptible stage was 7.1 x 10^4 spores ml^{-1} and 2.7 x 10^6 spores ml^{-1} for the

less susceptible stage. Lethal dose for second and third instar larvae treated with *M. anisopliae* were 8.9 x 10^5 and 3.2 x 10^6 conidia ml $^{-1}$, respectively.

Good control of *P. xylostella* larvae with *B. thuringiensis,* isolate Px- K3 and *M. anisopliae*, isolate ICIPE 20 was achieved in the green house experiment that compared favourably with one chemical pesticide, Lambda cyholothrin (Karate®). Isolate of *B. thuringiensis,* Px-K3 recorded the highest mortality of 96 % while Karate® exhibited the lowest mortality of 62 % at eight days post treatment.

The results obtained in this study suggest a potential for the deployment of these entomopathogens in the management of *P. xylostella.*

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CHAPTER ONE

1.0 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Cabbage (*Brassica oleraceae* var. *capitata*) is an important income generating vegetable crop in Kenya. It's popularity is due to the high demand for vegetables in the urban and export markets. The cultivation of this vegetable has therefore been intensified by small- scale farmers in central and western districts of the country (Oduor *et al.*, 1996). The crop is also an important diet for many Kenyans due to its value in vitamins and minerals but its production has been limited by many factors. The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera), has become the most important pest, causing massive destruction of cabbage and other crucifers worldwide (Madumadu *et al.*, 1991).

The management of this pest using chemical insecticides has not been successful. This approach is neither desirable nor sustainable. There is concern raised by consumers for insecticide residual - free cabbage. In addition, DBM has evolved resistance to all modern insecticides that have been used intensively for any length of time (Talekar and Shelton, 1993). It is therefore important to develop other pest management strategies that are safe, environmentally friendly and which can be used in integrated pest management systems. Pest control options involving the use of efficient native strains of entomopathogens like *B. thuringiensis* and *M. anisopliae* are likely to contribute to sustainable management of this pest.

There is need therefore, to isolate local strains of *B. thuringiensis* and *M. anisopliae* to evaluate their efficacy against *P. xylostella* in order to select the most potent isolates that could be utilized for use as biopesticides. The present study was therefore, carried out to investigate the possible use of local strains of *B. thuringiensis* and *M. anisopliae* in Kenya, as an alternative option to serve as part of the Integrated Pest Management (IPM) programmes being evolved for DBM.

The whole study encompasses five chapters. The first one outlines the objectives of the study and literature review on the diamond back moth and the entomopathogenic *B. thuringiensis* and *M. anisopliae.* Chapter 2 through 4 explores materials and methods, results and the general discussion. Chapter 5 accounts for a general conclusion and references. It also highlights some recommendations for future studies.

1.1.1 Justification

One of the major constraints to the production of cabbage in Kenya is crop loss resulting from DBM attack. Sustainable management of this pest has proved to be difficult since farmers mostly resort to repetitive and often indiscriminate use of chemical pesticides. The pest has developed resistance to nearly all-conventional pesticides (Magaro and Edelson 1990) and some non- conventional insecticides such as *B. thuringiensis* (Tabashnik 1994). The cost of DBM control has also increased due to pest resurgence as a result of destruction of their natural enemies by chemical pesticides. On the other hand, consumers are demanding for produce that is free from pesticide residues. This underscores the need, not only to rationalize and minimize pesticide use on cabbage crop, but also to explore safer alternatives. The screening for various local strains of *B. thuringiensis* and *M. anisopliae* could lead to useful alternative options for DBM control and so alleviate this escalating problem of pesticide use. The promising new strains of local pathogens could be deployed or used in commercial products that could be made available in the battle against DBM.

1.1.2 Hypotheses

1. Entomopathogenic *B. thuringiensis* and *M. anisopliae* occur naturally in different geographical regions in Kenya.
2. Pathogenicity of locally isolated *B. thuringiensis* and *M. anisopliae* varies with different isolates and different dose levels.

3. There are differences in the relative pathogenicity of local isolates of *B. thuringiensis* and *M. anisopliae* against different larval stages *P. xylostella*.
4. Local strains of *B. thuringiensis* and *M. anisopliae* are effective in controlling *P. xylostella* in the green house.

1.1.3 Objectives of the study

Studies were conducted to determine the potential of native isolates of *B. thuringiensis* and *M. anisopliae* for the control of *P. xylostella* in cabbage. This was achieved through the following specific objectives:

1. To isolate strains of entomopathogenic *B. thurigiensis* and *M. anisopliae* from Kenyan soils
2. To evaluate the efficacy of entomopathogenic *B. thurigiensis* and *M. anisopliae* on *P. xylostella*
3. To evaluate the susceptibility of various larval instars of *P. xylostella* to the selected isolates of *B. thuringiensis* and *M. anisopliae*.
4. To evaluate the potential of selected entomopathogenic *B. thuringiensis* and *M. anisopliae* for control of DBM in green house experiment.

1.2 LITERATURE REVIEW

1.2.1 Cabbage production in Kenya

Cabbages are grown throughout the year by small-scale farmers either under irrigation or rain-field conditions. The total area under cabbage in Kenya is approximately 30,000 hectares with a production capacity of 504,000 metric tones valued at 2 million dollars (MALDM, 1998).

Most town dwellers in Kenya do not have adequate land to grow vegetables and are dependant on regular supplies from the market (UNEP, 1995). Besides having significant nutritional value, cabbage crop also support many industries such as the cooking oil and fat processing industry, seed industry, fertilizer and plant protection, chemical industries, farm machinery and implements industry, packing and marketing industries (Veeraragavananthan *et al.*, 1998).

1.2.2 Constraints in cabbage production in Kenya

Despite the continued increase of production and acreage under cabbage crop, farmers are experiencing various constraints ranging from high production cost due to loses caused by pests and diseases (Tindall, 1983). Cabbage crop is often attacked seriously by arthropod pests. The intensity of these pests tend to be enhanced by inputs such as fertilizers and irrigation water resulting in more serious losses (ILACO, 1981).

Heavy infestation by DBM constitutes the single most prominent constraint for cabbage production in Kenya. It reduces yields by 30-40% on any crop it attacks especially

cabbages. In late growing season, it can cause losses of up to 100% (Le Van Trinh, 1996). Economic losses occur as a result of direct feeding damage to the cabbage, thus reducing the yields. In addition, physical presence of caterpillars within heads reduces the market value of cabbage. Furthermore, extra expenses are attributed to additional labour and increased use of insecticides.

1.2.3 Management of DBM in Kenya

Continuous cultivation of vegetables coupled with considerable inputs of fertilizers creates conditions that favour the build up of population of the vegetable pests (Lampkin *et al.*, 1994). Although selection of pest tolerant cabbage varieties and conservation of parasitoids are viable management tactics, it has been very difficult to provide economic control of pest without an effective insecticide. The high degree of efficacy, rapid kill and ease of use of broad spectrum chemical compounds like the pyrethroids have been a strong incentive to farmers to use them exclusively and as preventive measures to control pest populations (Osterman and Dreyer, 1995). Increased resistance to these insecticides in diamondback moth populations has necessitated increased dosages and more frequent applications of the insecticides. Moreover, in those regions where pesticide application is not closely monitored by central authorities, farmers often make a cocktail of the conventional insecticides or apply inappropriate chemicals in a desperate bid to contain the DBM problem (Talekar and Shelton, 1993).

The resulting reliance on this single approach to pest control has led to ever-increasing application rates, decreasing effectiveness and eventual breakdown of control. Many of the pesticides used are toxic and have long lasting residual effects (Wabule and

Ikitoo, 1997). There is therefore the risk of the produce carrying insecticide residuals above the maximum residue limits (MRLs), if selective use and pre-harvest waiting time are not implemented. This is especially true for the cabbage production sectors in Kenya, which are characterized by intensive use of insecticides so as to ensure adequate yields and produce damage- free vegetables for the urban markets.

1.2.4 The diamondback moth

The diamondback moth, *Plutella xylostella* (L.) is considered to have evolved on plants of the European family Brassicaceae and spread with cultivated brassicas around the world (Kfir, 1998). However, it now occurs wherever crucifers are grown. It is also believed to be the most universally distributed of all lepidopterans.

1.2.4.1 Life history

The adult moths are slender, measuring about 9 mm long (Harcourt 1960). They are greyish-brown with folded wings flaring outward and upward at their posterior ends. There are three pale triangular marks along the hind margin of each forewing, and when the wings are closed these marks form a diamond pattern, which gives the moth its common name (Plate 1a).

This species undergoes a complete metamorphosis. After pre-oviposition period of 2-3 days, the female moth may live a further 14 days and lay 50-150 eggs. Eggs are very small (0.5 to 0.25 mm), flattened base, pale yellow and are deposited singly or in clusters of 2 to 6, on the underside of the leave main veins (Plate 1b). The incubation period, which is influenced mainly by temperature lasts for 5-6 days (Talekar and Shelton, 1993).

The caterpillar is pale green, widest in the middle of its body, and it's about 12mm long when fully grown. There are four larval instars or stages, which are distinguished by the width of the head capsule. The duration of the four larval stages depends on temperature (Lu *et al.*, 1988). The total larval period varies from 14 to 28 days. The fourth instar constructs an open-network spindle shaped cocoon on the lower surface of the leaf in which it pupates. The pupa is greenish and the pupal period varies from 4 to 10 days depending on temperature (Chelliah and Srinivasan, 1986). Adults emerge after about 10 days (Pinley, 1975) and feed on water drops or dew. The number of generation per year varies depending on geographical area and seasonal conditions. In the tropics, breeding is continuous, with as many as 15 generations in one year.

1.2.4.2 Damage.

All larval instars are destructive but the greatest damage is caused by the third and fourth instars. The actual damage is as a result of larvae feeding on the outer leaves and mining into the center, damaging the young leaves (Plate 1c). The first instars mine in the leaf tissue and feed on the soft mesophyll tissue. Subsequent instars, however, are external feeders and consume all the tissue except the upper epidermis (Salinas, 1984), thus creating a window in the leaf. Early crop damage reduces the plants photosynthetic capacity, yields and may result in headless plants. Late damage may not affect yields but the perforated outer leaves lower the cosmetic quality and hence the market value of the crop. If not controlled, DBM can reduce the yields by 30 - 40% in a season (Le Van Trinh, 1996).

Plate1: Developmental stages of the Diamondback moth, *Plutella xylostella*

Plate 1a: Adult

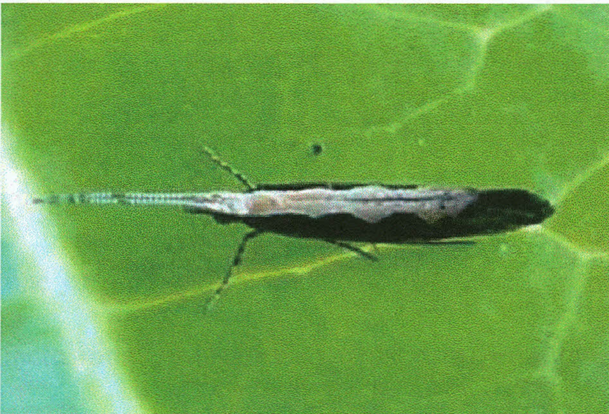


Plate 1b: Eggs

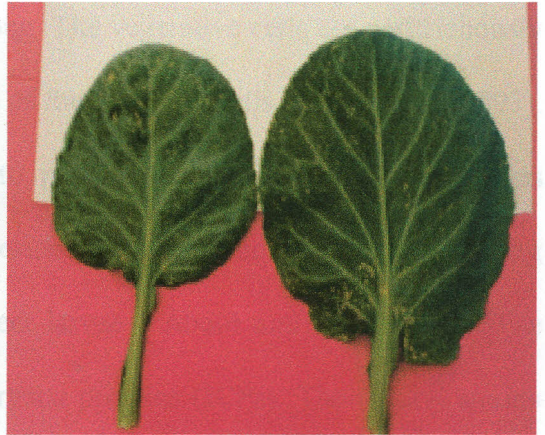
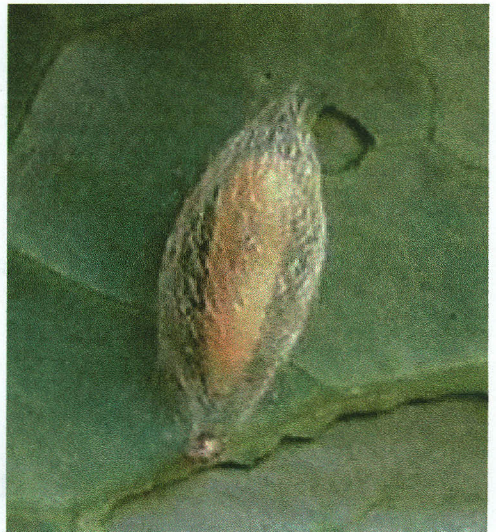


Plate 1c: Larva

Plate Id: Pupa



1.2.5 *Bacillus thuringiensis*

Bacillus thuringiensis is a rod-shaped, spore forming, gram -positive, aerobic bacterium. It measures 1.0 to 1.2 µm wide and 3 to 5µm long. The vegetative cells are peritrichously flagellated rods, which often occur as filaments of four or more cells (Luthy *et al.*, 1993). It is conventionally identified by its ability to produce parasporal crystalline bodies (Knowles, 1994). During sporulation, it synthesizes a cytoplasmic inclusion containing one or more proteins that are toxic to the insect larvae (Abdul and Ellar, 1999). The inclusions are also referred to as insecticidal crystal proteins (ICPs). The natural strains have been isolated from different sources, including grain dust, soil, insects and plants (Martin and Traver, 1989; Smith and Couche, 1991). An updated classification of *B. thuringiensis* strains is based upon flagella antigens or H-antigens (de Barjac and Frachon, 1990). Twenty-seven antigenic groups and seven subgroups have been distinguished (34 serovars) (de Barjac and Frachon, 1990).

Bacillus thuringiensis toxins are active against caterpillars, mosquitoes, beetles and nematodes (Hofte and Whitely, 1989). The protein crystal inclusions are classified into 22 classes referred to as Cry1 to Cry22 according to the similarity of the amino acid sequences (Crickmore *et al.*, 1998). The current studied crystal proteins can be classified into four major groups based on the specific activity and homology as well as protein structure.

CryI proteins are active against Lepidoptera, Cry II proteins against both Lepidoptera and Diptera, Cry III proteins against Coleoptera and Cry IV against Diptera. CryI proteins, such as CryIA (a), CryIA (b) and CryIA(c) are undoubtedly the most widely studied crystal proteins. CryI ICPs are all 130 to 140 kDa protoxins, which are processed into toxic, protease - resistant fragments of 60 to 70 kDa.

1.2.5.1. Mode of action of *B. thuringiensis* toxins

The delta-endotoxin of *B. thuringiensis* acts as insect gut poison and it is the major active component of commercial products (Fig 1). The toxic protein crystals of *B. thuringiensis* are only effective when ingested by insects with specific (usually alkaline) gut pH and the specific gut membrane structures required for binding the toxin (Hoffmann *et al.*, 1988).

When the susceptible insect ingests this bacterium, these crystals are solubilised in the alkaline environment of the insect midgut and processed proteolytically to yield smaller toxic polypeptides (Gill *et al.*, 1992). The toxin interacts with cells of the insect midgut epithelium via high affinity binding sites, disrupting the membrane integrity.

The Cry toxin is believed to bind specifically to receptor molecules in the midgut epithelium cells of host insect disrupting the ion permeability of the midgut cell membrane. This then induces formation of small specific pores in susceptible insect gut membrane cells resulting in a net influx of ions and accompanying influx of water, thus leading to swelling and lysis of the cells, causing spilling of their cytoplasmic contents into the lumen (Heimpel and Angus, 1959; Osir and Vundla, 1997).

Intoxication is also associated with immediate feeding inhibition due to damage of larval digestive tract. In other species changes in gut and haemolymph conditions permits vegetative propagation of *B. thuringiensis* within the insect, resulting in septicaemia, which contributes to or causes death (Haber and Luthy, 1981). Combination of gut leakage, lack of feeding and septicemia usually kills the insect within one to several days (Fast *et al.*, 1981) depending on the dose, species and environmental conditions

Figure 1. Mode of Action of *B. thuringeinsis* toxins

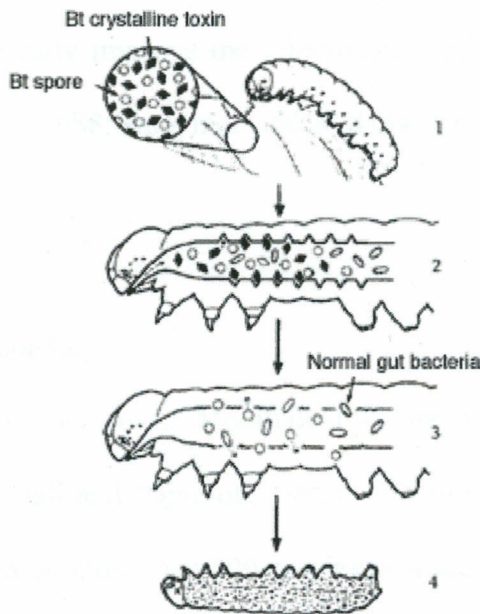


Diagram courtesy of **Abbott Laboratories**.

1.2.6 Fungal pathogens

Entomopathogenic fungi are common in the environment, particularly in the soil (Chandler *et al.*, 1997). They often decimate insect populations in spectacular epizootics. The most important species are *Beauveria bassiana, Metarhizium anisopliae, Paecilomyces farinosus, P. fumosoroseus* and *Verticillum lecanii.* Insect pathogenic fungi are very specific and do not affect other animals and plants (Hoffmann, 1993). Several fungi have been isolated from *P. xylostella* (Humber, 1992). More recently, (Shelton *et al.*, 1998) and (Vandenberg and Ramos, 1998) have shown that commercial formulations of *B. bassiana* and *M. anisopliae* can control *P. xylostella* populations on crucifer seedlings in screen houses and large plants in the field. Rigorous strain selection and oil-based formulations can greatly improve the effectiveness of insect pathogenic fungi in dry ecosystems (Prior *et al.*, 1988; Kooyman and Abdalla, 1998).

1.2.6.1 *Metarhizium anisopliae*

Metarhizium (Deuteromycetes: Moniliales) is the most ubiquitous species of entomopathogenic fungi (Hall and Papierok, 1982). This genus is responsible for green muscardine disease known on more than 200 species of insects. Two species are known, *M. anisopliae* (Metschnikoff) Sorokin and *M. flavoviride* Gams and Rozspal. It can be isolated easily from the soil using selective media and '*Galleria* bait' technique (Zimmerman, 1986). It is postulated that the most virulent isolates are those derived from the target insects affected under natural conditions (Glare and Milner, 1991). Several insect pests, including termites, locusts, spittlebugs, cockroaches and beetles are being

targeted for control by *M. anisopliae* (Zimmerman, 1993). *Metarhizium anisopliae* is a fungal entomopathogen, which under favourable environmental conditions, can cause natural epizootics in *P. xylostella* populations and show promise as a biological control agent (Furlong *et al.*, 1995). Although the pathogen can cause mycosis in *Diadegma semiclausum* (Furlong and Pell, 1996), one of the principle natural enemies of *P. xylostella*, (Syed *et al.*, 1997), the susceptibility in the laboratory is unlikely to translate into susceptibility in the field (Furlong and Pell, 1996).

1.2.6.2 Mode of action

Metarhizium anisopliae, as most of fungal pathogens, initiates infection by adhesion of the conidium on the host cuticle. Attachment is normally achieved through the secretion of adhesive mucus as the conidium swells during pregermination development, which supplements the initial hydrophobic interactions between the conidium and the cuticle surface. However, enzymes, lectins, hydrophobic bonding and electrostatic forces also play a role (Boucias *et al.,* 1982). Musilage associated with some fungi interacts with and modifies epicuticular waxes as indicated by production of imprints of invasive propagules on the cuticle (Wright *et al.,* 1990). This is achieved by action of cuticle degrading enzymes (CDEs) secreted with the mucilage and probably serves in the host recognition as well as cementing the pathogen to suitable substratum (Butt, 1990).

Conidium then germinates on the insect cuticle after attachment. Various factors are vital for spore germination such as water, ions, fatty acids, nutrients, the biota on the cuticle surface, and the physiological state of the host (Butt, 1990). Generally, high humidity is needed for germination but the microenvironment on the host cuticle and foliage, particularly during periods of dew, frequently afford the proper conditions for germination even when the macroclimate is very dry. Hassan *et al.*, (1989) have shown that soaking conidia of *M. anisopliae* accelerates germination and these spores are more virulent towards *Mandusca Sexta* L.

Cuticular invasion involves both enzymatic and physical activities. The enzymes elaborated by germinating conidia have not been identified, but is known that colonies of entomopathogenic fungi such as *B. bassiana, B. brongniartii, M. anisopliae* and *Entomophthora muscae* produce protease, lipases and chinases in liquid and agar media (Gabriel, 1968). *Metarhizium anisopliae* penetrates the cuticle by development of appressoria (located at the cuticle surface), infection pegs (in the epicuticle), penetrant hyphae and penetrant plates (in the procuticle) and yeast like bodies (blastospores) for dispersal through the haemocoel (Hajek and Leger 1994). This series of events is represented schematically in Figure 1.

After crossing the cuticular barrier, the fungus then overcomes the cellular defense reaction of the host by production of toxins. The cyclic depsipeptide destruxins are produced by isolates of *M. anisopliae* in amounts that correlates the toxicosis and differential virulence of isolates against some insects (Charnley *et al.*, 1988). Destruxins

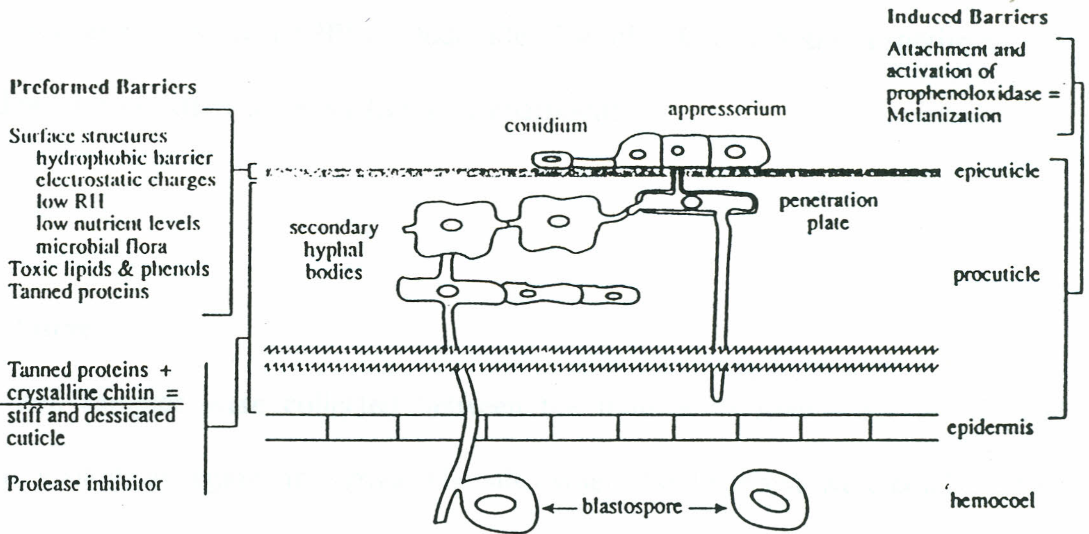
affects various organelle targets (such as mitochondria, endoplasmic reticulum and nuclear membranes) paralysing cells and causing disfunction of the mid- gut, malphigian tubules, haemocytes and muscles.

The host may be killed by a combination of mechanical damage produced by fungal growth, nutrient depletion and toxicoses (Gillespie and Claydonn 1989). Death of the insect marks the end of the parasitic phase of the fungus. This may be preceded by behavioural changes such as cessation of feeding, weakness and disorientation (Ferron, 1978). The host often changes colour and may show dark spots indicating areas of fungal infection.

After the death of the host, the fungus proceeds to grow saprophytically and spread through virtually all tissues of the insect. Cadavers are transformed into mummies resistant to bacterial decay apparently because of antibiotics produced by the fungus. The fungus is conserved in the form of chlamydospores if the 'mummy' is held under conditions of moderate to low humidity.

When the atmosphere reaches saturation the mycelium emerges through the integument and develop conidiophores (Ferron, 1978). Infective unit occurs on the exterior of the host insect and each cadaver constitutes an infective focus, which by multiplying of the quality of inoculum ensures propagation of the disease. Spores are dispersed by various agents such as air, rain, and even insects and mites (Ferron, 1978).

Figure 2: Schematic representation of infection structures of *Metarhizium anisopliae* and cuticular resistance barriers in cross-section of insect cuticle. (Hajek and Leger 1994)



CHAPTER TWO

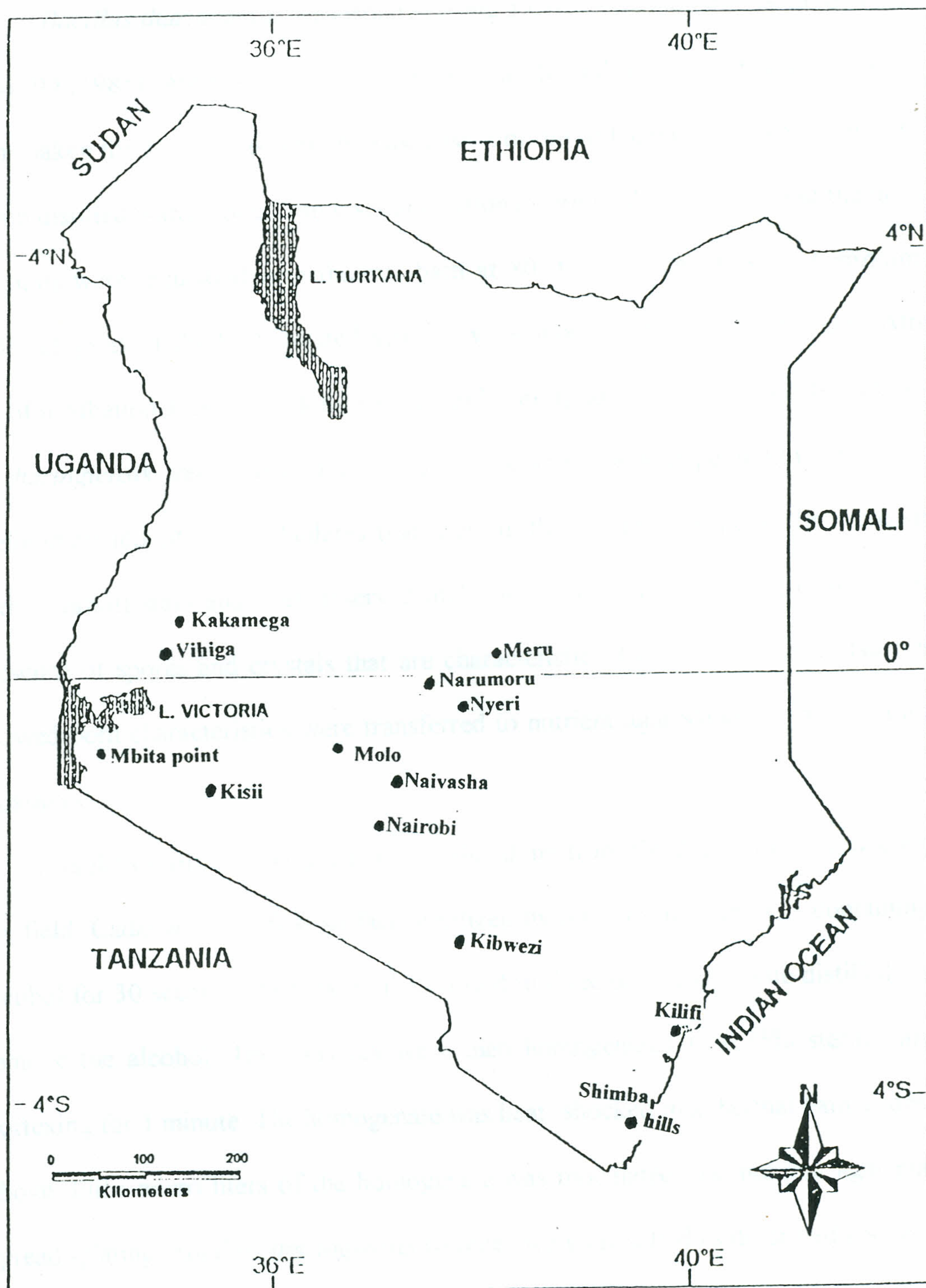
2.0 Materials and methods

Laboratory studies were conducted at the International Centre of Insect Physiology and Ecology (ICIPE), Duduville, Nairobi. Green house experiment was carried out at Kenyatta University Botany Department.

2.1 Survey

Soil samples were collected between the months of June - July 2000 from different ecological regions in Kenya. The sites where field surveys were conducted are shown on the map of Kenya (Figure 3). The samples were collected randomly from different sites in each region. A 1 m^2 area was chosen and 2 sub samples of approximately 50g were taken from up to 10 cm deep in each site. The sites were numbered and sampled repeatedly throughout the study. Soil samples were kept in sterilized vials and taken to the laboratory for isolation of *B. thuringiensis* and *M. anisopliae*. A total of 60 soil samples were taken during the survey. Diseased/dead insects were collected from the field. Healthy larvae were also collected, brought to the laboratory and observed for any disease for one week.

Figure 3: Map of Kenya showing survey sites



2.2 Isolation and cultivation of *B. thuringiensis*

Bacillus thuringiensis was isolated using a modified method of isolation described by WHO (1985). Approximately 10 g of soil was diluted in 90 ml of saline solution and then shaken for 30 minutes. One-ml suspension of the soil sample was added into 9 ml of sterile distilled water. Successive serial dilutions were made up to 10^{-6} and the last three dilutions were incubated in a thermal bath at 80° C for 15 minutes. Approximately 100μl aliquots of the heat- treated samples were inoculated into nutrient agar. After 24 hrs of incubation at 30° C, colonies with similar morphological characteristics to those of *B. thuringiensis* were transferred to nutrient agar containing penicillin (100 ml/l) for preliminary identification. Isolates that grew in the presence of penicillin were stained with Smirnoff stain and later observed under microscope (Mg x 1000) to determine the presence of spores and crystals that are characteristic of *B. thuringiensis.* Isolates that showed both characteristics were transferred to nutrient agar slants for preservation and bioassays.

Isolation of *B. thuringiensis* was also done from the dead insects collected from the field. Cadavers were first surface sterilized by dipping them in vials containing 70% alcohol for 30 seconds. They were then rinsed in three bottles of sterile distilled water to remove the alcohol. The cadavers were then homogenized in 0.85% sterile saline by vortexing for 1 minute. The homogenate was heat- shocked in a thermal bath as described above. Fifty micro liters of the homogenate was inoculated into nutrient agar plates by spread -plating. *Bacillus thuringiensis* colonies were identified as described above.

2.3 Microscopy and staining procedures:

Staining of *B. thuringiensis* cells was carried out as described by (Lisansky *et al.* 1993). Three drops of solution A (1.5 gm Amido black dissolved in 50 parts of acetic acid) was added to the heat fixed slides of *B. thuringiensis* cells and washed off after 70 seconds. Thirty percent (30%) of solution B (1gm of basic Fuchsin dissolved in 10 ml of 95% ethanol and mixed with 5 gm of phenol dissolved in 90 ml of distilled water) was then added to the slide and left for 20 seconds before washing in cold tap water and drying on the filter paper.

2.4 Isolation and cultivation of *M. anisopliae*

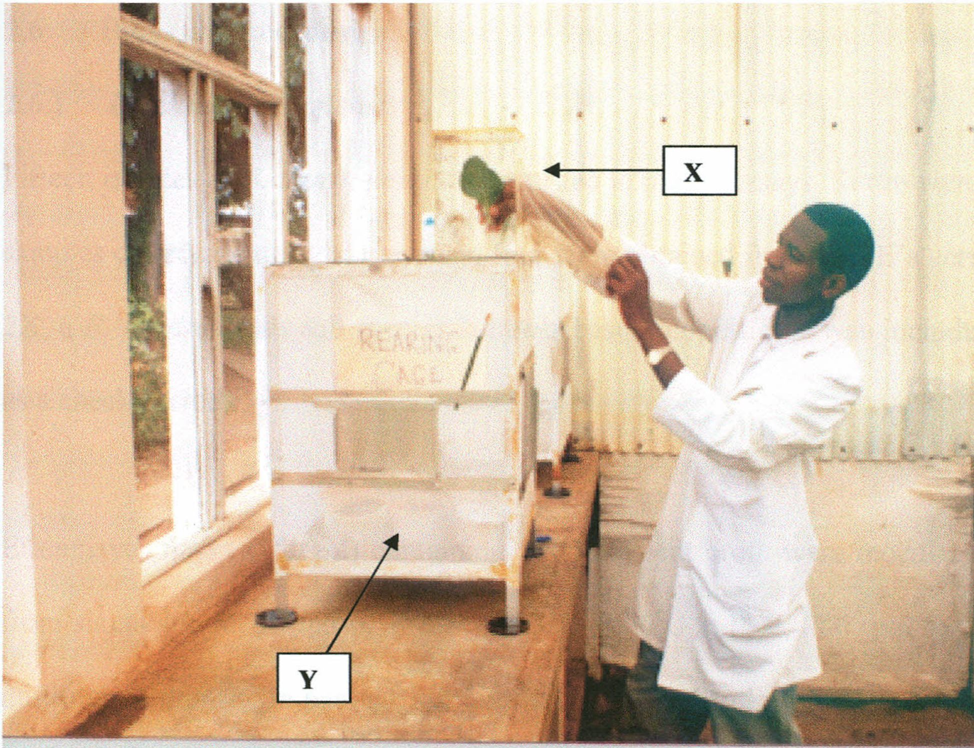
Ten grams (10g) of soil were added to 90 ml of sterile distilled water and homogenised by shaking for one hour to release fungal propagules from the soil. Aliquots (100µl) were spread-plated on selective agar media (Veens & Ferron media) by swirling the Petri dishes. The cultures were incubated for 3-7 days at 25 ± 2°C. Colonies of *M. anisopliae* that developed on selective media were transferred to Sabouraud Dextrose agar media supplemented with 1% yeast extract (SDA + YE) to obtain pure cultures.

Isolation of *M. anisopliae* from dead insects was done by first surface sterilizing the cadavers in 70% alcohol solution for 30 seconds and rinsed in three bottles of sterile distilled water. Larvae were then transferred aseptically to Petri dishes lined with damp filter paper. The Petri dishes were then sealed with parafilm and maintained at a temperature of 26 ± 2°C. Observation was made after 3-4 days for the presence of mycoses typical of *M. anisopliae*

2.5 Insect culture

The initial stock culture was obtained from the field population of diamondback moth, *P. xylostella* infesting cabbage plants in Kiambu district. In order to have a regular supply of larvae of the same age, the culture was maintained in a controlled environment room at temperature of 25 ± 2°C, and relative humidity (RH) of 75 ± 5%, under a photo period of 12h L: 12h D. The adult moths were released into small oviposition cages (30 x 30 x 30 cm) (Plate 2). The leaf petiole was immersed in a vial containing water to prevent it from withering. Adult moths were allowed to lay eggs on the leaf. The leaf was removed after 24 h and placed in a Petri dish lined with a filter paper. The Petri dish containing eggs was sealed with a parafilm to maintain the moisture inside and to prevent the newly hatched larvae from escaping. The eggs hatched within 2-3 days and the newly hatched larvae were provided with fresh leaves until required for bioassays. Some larvae were maintained on potted cabbage plant inside the rearing cages for the continuity of the culture. The larval period took about three weeks. Pupation occurred on the underside of the leaves. Generally the whole cycle was accomplished after four weeks.

Plate 2: Rearing of the diamondback moth



Key

X ⟶ **Oviposition cage**

Y ⟶ **Rearing cage**

2.6 Bioassay procedures.

2.6.1 *Bacillus thuringiensis*

Fifteen isolates of *B. thuringiensis* were tested in the bioassays. Seven were isolated from samples collected during surveys and four were obtained from ICIPE's germplasm. Dipel 2X, a *B. thuringiensis* sub species *kurstaki* based biopesticide was included in the assay as a check period.

Erlenmayer flasks (150 ml) containing 50ml nutrient broth were inoculated with culture from slants and incubated in a rotary shaker at 30°C ± 1^0 C, 200 rpm for 24 hours. One milliliter of this single passage seed culture was used to inoculate 100 ml nutrient broth for 72 hours at 30°C ± 1^0C.

2.6.1.1 Screening assay

Two hundred micro-liters of the broth culture of each isolate were diluted in 10 ml of sterile distilled water containing 15%v/v glycerol. This was chosen as a standard dose for screening experiments. This suspension was sprayed on leaf discs of 5 cm diameter the Burgerjon - spray tower. The control was treated with sterile distilled water. The treated leaf discs were placed into Petri dishes and allowed to air dry for 15 minutes. Twenty first- instar DBM larvae were transferred to the Petri dishes lined with moistened filter papers containing the treated leaf discs. Each treatment was replicated four times.

Mortality was recorded daily for 4 days. Mortality caused by *B. thuringiensis* was confirmed by examination of the *B. thuringiensis* colonies after plating the cadavers on nutrient agar plates for 24 h at 30°C.

2.6.1.2. Dose - mortality response experiments

Following the screening, four *B. thuringiensis* isolates, MR-1, KF, NA-3 and Px-K3 were found to be highly pathogenic accounting for 100% mortality in 2 days. Thus, they were selected for dose mortality relationship experiments.

Twenty millilitres of broth culture of the selected isolates were centrifuged at 4°C, 8000 rpm for 30 minutes in a Sorval GSA rotor, model RC- 5C Sorval centriguge (Du pont, Delware, USA). The supernatant was discarded aseptically and the resulting pellet was washed twice in sterile distilled water. The concentrated pellet of spore crystal complex was resuspended in 10 ml of sterile distilled water containing 15%v/v glycerol solution and stored at -20°C and used as necessary. The number of the spores/ml of each stock suspension was calculated using an improved Neubauer haemocytometer following a procedure described by Baker and Silverton (1980).

The effect of dose on mortality of 1st instar larvae of *P. xylostella* was determined by exposing larvae to three dose levels; $1x10^5$, $1x10^6$, and $1x10^7$ spores/ml. The treatments were performed by spraying 10 ml of a given concentration of *B. thuringiensis* onto the cabbage leaf discs using the Burgerjon's spray tower. Control lots were treated with 10ml of sterile distilled water containing 15%v/v glycerol. The treated leaf discs were placed

into Petri dishes and allowed to air dry for 15 minutes. The experiment consisted of four replicates of 20 larvae for each dose. Mortality was recorded daily for 4 days.

2.6.1.3. Larval age response to bacterial infection

The susceptibility of three *P. xylostella* larval instars to *B. thuringiensis* isolate Px-K3 was determined by using three dose levels of $1x10^5$, $1x10^6$, and $1x10^7$ spores/ml. Second, third and fourth- instar larval stages were used. Ten milliliters (10ml) of a given dose was used to treat leaf disc using the Burgejon's spray tower as described earlier.

2.6.1.4. Recovery of *B. thuringiensis* from dead larvae.

Some of the dead larvae were dissected on microscopic glass slides using a sterile dissecting needle. The haemolymph was streaked onto a nutrient agar plate using a sterilized inoculating loop and then incubated at 30° C. Other dead larvae were mounted directly on a nutrient agar plate and the growth of *B. thuringiensis* observed after 24 hours.

2.6.2 *Metarhizium. anisopliae*

Fourteen isolates of *M. anisopliae* were tested. Eleven isolates were obtained from the ICIPE culture collection while the other three; MR-1, NA-3 and KZ-3 were isolated from the soil samples collected during the survey (Table 1). Fungi were cultured on SDA plates and maintained at 25 ± 2°C in the incubator.

2.6.2.1 Preparation of the inoculum

Conidia were harvested by scrapping the culture surface of 21 day - old culture. Conidia were suspended in 20 ml sterile distilled water containing 0.05% Triton X-100 in universal bottles containing 3 mm glass beads. The conidial suspension was vortexed for 5 minutes to produce a homogenous suspension. Conidial concentrations were determined using a haemocytometer.

2.6.2.2 Viability test

The viability of the conidia were determined by spread - plating 0.1ml of conidial suspension titrated to $3x10^6$ conidial ml^{-1} on the SDA plates. A sterile microscope cover slip was placed on each plate. The plates were examined after 24 hours. The percentage germination was determined from 100-spore count on each plate on each cover slip. Each plate was replicated four times.

2.6.2.3. Screening

All the fungal isolates were screened at a standard concentration of $1x10^7$ conidia ml^{-1}. The treatments were performed by spraying 10 ml of conidial suspension onto the leaf discs using Burgerjon tower (1956). The leaf discs were sprayed with sterile distilled water containing 0.05% Triton X-100 for control treatments. The discs were allowed to air dry for 15 minutes. Twenty first- instar larvae of DBM were transferred onto leaf disc, which was placed in a Petri dish with moistened filter paper at the base. The experiment consisted of 4 replicates of 20 first instar larvae for each strain. Mortality was recorded daily for 8 days.

2.6.2.4. Dose- mortality response experiments

Two strains of *M. anisopliae*, IC20 and KZ-4 were found to be virulent during the screening, causing 100% mortality within 5 days. They were therefore considered for dose- mortality response experiments.

First- instar larvae of DBM were treated with five concentrations of inoculum: 0, $1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1} using the Burgerjon spray tower. Twenty 2-day-old larvae were used and the experiment was replicated four times. The other conditions of the experiment remained the same as described earlier.

2.6.2.5. Larval age response to infection to *Metarhizium anisopliae*

Isolate IC20 was tested against second and third - instar larval stages of *P. xylostella*. Three dose levels of $1x10^6$, $1x10^7$ and $1x10^8$conidia ml^{-1} were used against each stage by spraying 10 ml of a given concentration of spores onto leaf discs using the Burgerjon's spray tower. Control lots were treated with sterile distilled water containing 0.05% Triton X-100. Twenty larvae were then transferred onto each treated leaf disc. The experiment consisted of 4 replicates of 20 insects for each concentration at each larval stage. Treated larvae were placed in Petri dishes and placed in humid plastic containers at 26 ± 2°C. Mortality was recorded daily for 7 days.

2.6.2.6. Recovery of *M. anisopliae* from dead larvae

Dead larvae were surface- sterilized in 70% alcohol solution for 30 seconds and rinsed three times in sterile distilled water. Larvae were then transferred aseptically to Petri dishes lined with damp filter papers, sealed with parafilm and maintained at a temperature of 26 ± 2°C. The mortality caused by the fungi was confirmed by microscopic examination of the hyphae and spores on the surface of the cadavers.

2.7 Effect of *B. thuringiensis* isolate, Px-K3 and *M. anisopliae*, isolate IC20 in green house

Virulence of the *B. thuringiensis* strain Px-k3 and *M. anisopliae* strain IC20 was evaluated in the green house at Kenyatta University Botany department. This test was done following of a modification of a method described by Yoon *et al* (1999). Cabbage variety Copenhagen was planted in pots with a diameter of 15 cm (Plate 3). The plants were left to grow up to the sixth week. Cabbage plants were artificially infested with DBM eggs. The Cabbage leaves bearing 2-day-old eggs were transferred onto the potted cabbage plants. Approximately 50 eggs were attached onto the adaxial side of the leaves in each plant and the eggs left to hatch. The entomopathogens were applied in aqueous form at a standard concentration of $1x10^7$ conidia ml^{-1} for *M. anisopliae* and $1x10^7$ spores ml^{-1} for *B. thuringiensis*. Using a hand sprayer, the concentrations were sprayed to each of 5 replicated plants. Control plants were treated with sterile distilled water. A synthetic insecticidal treatment lambda cyhalothrin (Karate®) and a commercial product, Dipel

2X® (*B. thuringiensis* sub species *kurstaki*) was used as checks. The physical properties of the formulations were followed as described by the manufacturer. Karate was applied at a rate of 0.25 ml per liter of water while 0.5g were dissolved in water incase of Dipel 2X®.

The number of live larvae on each plant was recorded at 0, 2, 4 and 8 days after the spray. Physical leaf damage caused by defoliating larvae was determined by estimating the extent of leaf defoliation using an adaptation of Ogol and Spence (1997) empirical scale. (Table1).

Table 1. Insect damage rating scale (Ogol and Spencer, 1997)

Rating	Damage percentage rating	Relative description of severity
1	<10%	No damage
2	11-25%	Light damage
3	26-50%	Moderate damage
4	51-75%	Severe damage
5	>76%	Very severe damage

Plate 3: Greenhouse Experiment for evaluating the effectiveness of biopesticides and a chemical pesticide against *P.xylostella*

Plate 3 a: Experimental set up

Plate 3 b: Attachment of DBM Eggs on Adaxial side of Cabbage leaves.

2.8. Data Analysis

The recorded percentage mortality was normalized through angular transformation after adjusting for natural mortality in controls using Abbott's (1925) formulae. Mortality rates were separated across different isolates and concentrations with Student-Newman Kuels (SNK) test (P=0.05), using the ANOVA procedure of SAS. Lethal time (LT_{50}) and lethal concentration (LC_{50}) values were determined for each isolate using the probit analysis method of SAS.

Data collected on scores (as percentage of damage) on foliar damage (as counts) was subjected to square root transformation and analyzed using two way factor ANOVA. Means were separated using the SNK test.

CHAPTER THREE

3.0 RESULTS

3.1 Isolation of *B. thuringiensis* and *M. anisopliae* from the soil and cadavers

Eleven strains of *B. thuringiensis* were isolated from the samples collected from the surveys. Ten of the isolates: KZ-3, SH-4, MR-1, NA-3, NA-2, ML-4, KF-2, SH-4, MP-3, SN-5 and NY-2 were isolated from the soil samples and one, Px-K3, from dead larvae of *Plutella xylostella* (Table 2)

On the other hand, strains *M. anisopliae* were isolated from soil samples (Table 3).

Table 2. Isolates of *B. thuringiensis* isolated from samples

ISOLATE CODE	SOURCE	LOCALITY
KZ-3	Soil	Kibwezi
SH-4	Soil	Shimba hills
MR-1	Soil	Meru
NA-2	Soil	Naivasha
NA-3	Soil	Naivasha
ML-4	Soil	Molo
KF-2	Soil	Kilifi
MP-4	Soil	Mbita point
SN-4	Soil	Suneka (Kisii)
NY-4	Soil	Nyeri
Px-K3	*P. xylostella*	Kijabe.

Table 3. *Metarhizium anisopliae* isolates screened against first instar *P. xylostella* larvae.

ISOLATE	SOURCE	LOCALITY	YEAR
IC18	Soil	Kenya	1994
IC20	Soil	Migori (Kenya)	1989
IC30	soil	Unkown	1990
IC60	Soil	Kinshasa (DRC	1990
IC69	Soil	Mbita point	1990
IC63	Soil	Kinshasa (DRC)	1990
IC75	*C. punticolis*	Mbita point	1990
IC78	*T. nigroplagiata*	Ungoye	1990
CAT1	*S. exempta*	Machakos	2000
K4	Soil	Kitui	2000
KZ-4	Soil	Kibwezi	2000
MR-1	Soil	Meru	2000
NA-3	Soil	Naivasha	2000

3.2.0 Pathogenicity of *M. anisopliae* and *B. thuringiensis* isolates against *P. xylostella* larvae.

In viability tests, germination of conidia varied between 65-100 % after 24 hrs post exposure (Figure 4). *Plutella xylostella* larvae that died from *M. anisopliae* treatments rapidly exhibited mycosis on the surface of cadavers under moist conditions. The emergent hyphae from the cadaver was from the intersegmental regions but covered the entire cadaver within a few days with diffuse hyphal growth. On the other hand, all *B. thuringiensis*-treated larvae showed characteristic growth of the bacterial cells after 24h at 30°C in nutrient agar plate (Plate 5). *Bacillus thuringiensis* cells also grew in the nutrient agar inoculated with the homogenate derived from the cadavers.

Figure 4. Viability of conidia of *M. anisopliae* isolates after 24 h on SDA plates at 26± 2°C

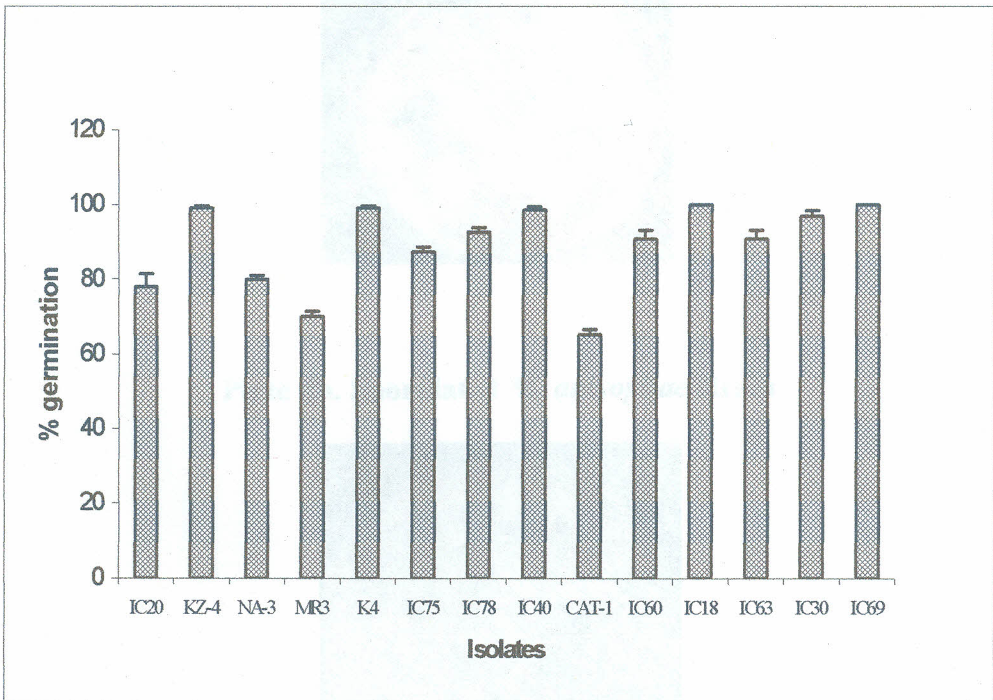


Plate 4: Cultures of M. anisopliae isolate grown on Sabouraud Dextrose agar Media (SDA)

Plate 4a. Single colony forming units of *M. anisopliae*

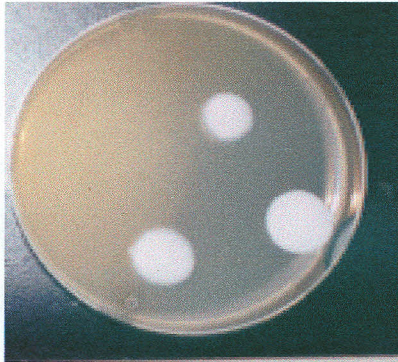


Plate 4b. Diffused hyphal growth on SDA Media

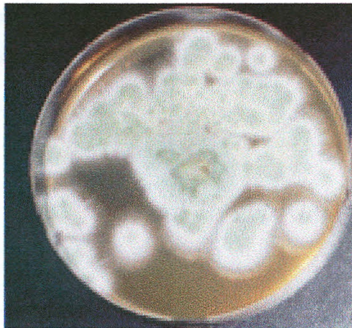


Plate 4b. Sporulated *M. anisopliae* strain

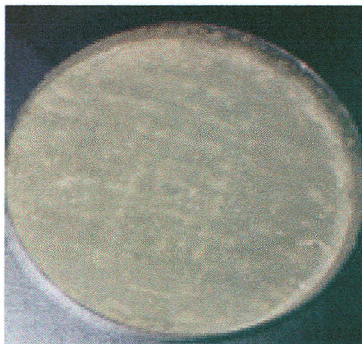
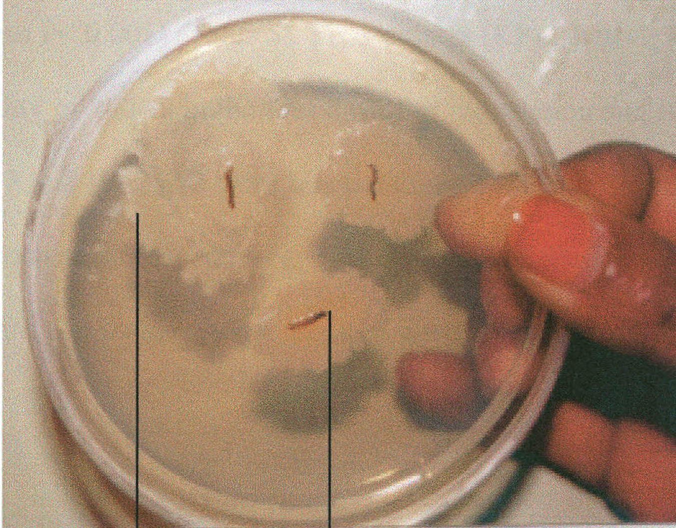


Plate 5: Bacteria recovered from a cadaver treated with *B. thuringiensis* isolate Px-K3



3.2.1 Bacillus thuringiensis

Mortality caused by *B. thuringiensis* isolates to the 1st instar larvae of DBM varied between 6 and 100 %. There were significant differences in the pathogenic activity between the isolates P< 0.001) (Table 5). Five isolates (KF-2, MR-1, NA-2, NA3 and Px-K3) caused mortality of 100 % in the first day after inoculation and two isolates within 4 days. Isolates B.t 3 and *B.t* 252/2 were less pathogenic killing 6.3 and 25% DBM larvae four days after inoculation respectively. The LT_{50} also differed significantly P<0.001). The LT_{50} values for the most virulent isolates were 0.5 days and 8.3 days for the less virulent (Table 5).

3.2.2 Metarhizium anisopliae

All the fourteen isolates of *M. anisopliae* were pathogenic to DBM larvae. Mortality ranged between 40-100% (Table 6). However, pathogenicity differed with the isolate. There were significant differences among the isolates at seven day post-inoculation P< 0.001). The onset of all of mortality was observed from third and fourth day after treatment in all of the isolates and increased afterwards. Isolates IC20 and KZ-4 were the most pathogenic causing 100% within 5 days. Isolate IC30 was the least pathogenic and caused mortality of 40% in 8 days. Lethal time values were also significantly different among isolates P<0.001). *Metarhizium anisopliae,* isolate IC20 had the shortest LT_{50} of 2.3 days and isolate IC30 had the longest LT_{50} of 8.1 (Table 6). The cabbage leaf damage was significantly reduced on leaf disc treated with IC20 than other *M. anisopliae* isolates.

Table 4. Pathogenicity of *Bacillus thuringiensis* isolates against 1st -instar larvae of *Plutella xylostella* following treatment of cabbage leaf discs at the concentration of 10^7 spores ml^{-1}. Mortality and LT_{50s} recorded 4 days post inoculation.

B. thuringiensis isolate	Percentage mortality ± S.E.	LT_{50} (± S.E.)
Bt252/2	25 ± 2.0d	5.24 ± 0.17b
Bt3	6.3 ± 1.3e	8.33 ± 0.6a
HD220	40 ± 2.0c	4.16 ± 0.1cd
KF-2	100a	0.83 ± 0.1f
KZ-3	100a	0.89 ± 0.89f
M37	100a	1.56 ± 0.08f
ML4	35 ± 2.0cd	4.77 ± 0.5cb
MP4	100a	1.38 ± 0.3f
MR1	100a	0.50 ± 0.0f
NA2	100a	0.50 ± 0.0f
NA3	100a	0.50 ± 0.0f
NY4	42.5 ± 3.2c	3.98 ± 0.14cd
Px-K3	100a	0.50 ± 0.0f
SH4	88.7 ± 4.3b	2.42 ± 0.13e
SN4	46.3± 3.8c	3.70 ± 0.17d

Means in the same column followed by the same letter are not significantly different at $P< 0.05$ by Student-Newman-Kuels.

Table 5. Pathogenicity of *Metarhizium anisopliae* isolates against 1st -instar larvae of *Plutella xylostella* following treatment of cabbage leaf discs at the concentration of 10^7 conidia ml^{-1}. Mortality and LT_{50s} recorded 7 days post inoculation.

Isolate	Percentage mortality (± S.E.)	LT_{50} (± S.E.) (Days)
CAT1	47.5 ± 3.2f	7.4 ± 0.33b
IC18	67.7 ± 1.4e	5.9 ± 0.14c
IC20	100a	2.3 ± 0.09g
IC30	40 ± 2.0f	8.1 ± 0.28a
IC40	100a	4.1 ± 0.07d
IC60	70.5 ± 2.0e	5.7 ± 0.19c
IC63	100a	3.8 ± 0.15de
IC69	82.5 ± 3.2c	5.5 ± 0.18c
IC75	82.5 ± 3.2c	5.6 ± 0.09c
IC78	78.7 ± 2.4dc	5.6 ± 0.23c
K4	100a	5.9 ± 0.2c
KZ-4	100a	2.8 ± 0.07f
MR-3	100a	3.2 ± 0.14ef
NA-3	100a	3.2 ± 0.15ef

Means in the same column followed by the same letter are not significantly different at $P < 0.05$ by Student-Newman-Kuels.

3.3 Effects of different concentrations of *Bacillus thuringiensis* and *Metarhizium anisopliae* on mortality of *Plutella xylostella*

Based on the results on time- mortality relationships, four isolates of *B. thuringiensis* (KF-2, MR-1, NA-2 and Px-K3) and two of *M. anisopliae* (ICIPE 20 and KZ-4) were selected for dose-mortality responses against first instar *P. xylostella* larvae.

Three doses were used: $1x10^7$, $1x10^6$ and $1x10^5$ spores/ml. Mortality of larvae of *P. xylostella* exposed to different concentrations of *B. thuringiensis* was dose-dependent (Figure 4a). For example, at lowest dose of $1x10^5$ spores ml^{-1}, *B. thuringiensis* isolates KF-2, MR-1, NA-3 and Px-K3 caused mortality of 45, 40, 59 and 74 % respectively. At the highest concentration of $1x10^7$sporesml^{-1}, isolates NA-3 and Px-K3 caused mortality of 100% while the other two isolates KF-2 and MR-1 caused mortality of 98 and 99 %, respectively. The LD_{50} values for the four isolates are shown in table 7. There was no significant difference in the LD_{50} for all the *B. thuringiensis* isolates

Similar trend was observed with the two *M. anisopliae* isolates, tested at the concentrations of $1x10^7$, $1x10^6$ and $1x10^5$ and $1x10^8$ conidia ml^{-1}. Mortality was also dose - dependent (Figure 6 & 7). However, there was a significance difference between both isolates at concentrations of $1x10^6$and $1x10^7$ conidia ml $^{-1}$. The LD_{50} values for both strains are shown in table 7.

Table 6. Probit-mortality-dose regression parameters for *P. xylostella* instars treated with four different *B. thuringiensis* isolates

Isolate	LD_{50} and 95% fiducial limits (Spores ml^{-1})	Slope ± SE[1]
KF-2	5.1×10^5 (4.3×10^5 - 6.2×10^5)	2.07 ± 0.14
MR-1	2.3×10^5 (1.9×10^5 - 2.8×10^5)	2.16 ± 0.18
NA-3	1.5×10^5 (1.2×10^5 -1.7×10^5)	2.54 ± 0.21
Px-K3	1.2×10^5 (0.1×10^5- 1.4×10^5)	2.66 ± 0.24

[1]SE, standard error of slope

Table 7. Probit-mortality-dose regression parameters for *P. xylostella* instars treated with two different *M. anisopliae* isolates

Isolate	LD_{50} and 95% fiducial limits (conidia ml^{-1})	Slope ± SE[1]
IC20	2.3×10^5 (1.9×10^5- 2.8×10^5)	1.86 ± 0.14
KZ-4	3.9×10^5 (2.2×10^5 - 8.7×10^5)	0.56 ± 0.08

[1]SE, standard error of slope

Figure 5. Effect of different concentrations of *B. thuringiensis* on *P. xylostella* larvae at 2-day post inoculation.

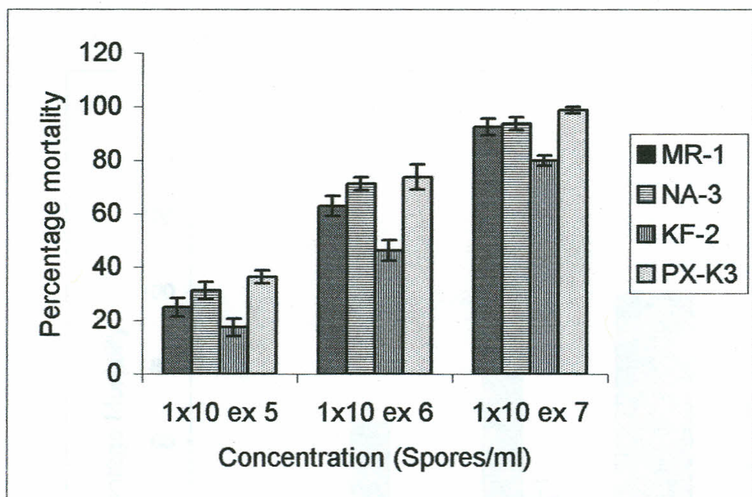


Figure 6. Effect of different concentrations of *M. anisopliae* on *P. xylostella* larvae at 3 -day post inoculation.

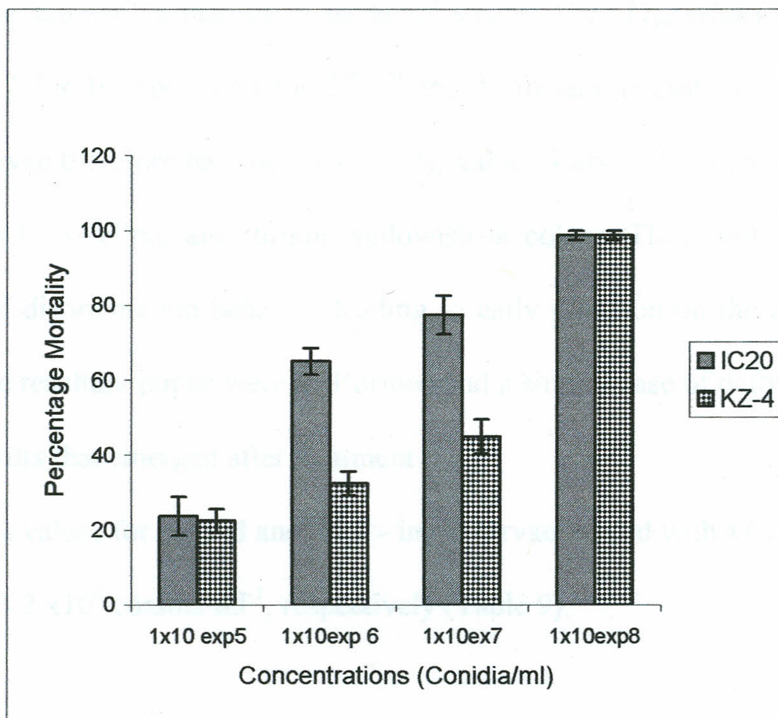
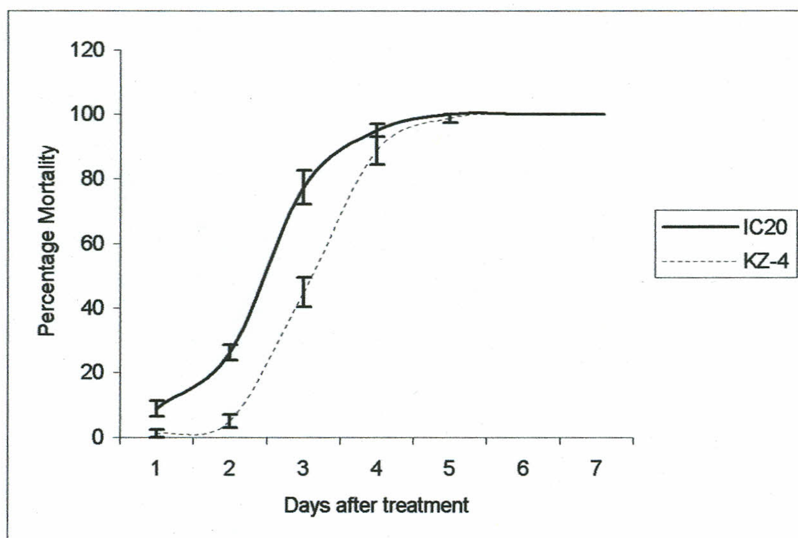


Figure 7. Mortality of first-instar larvae *P. xylostella* exposed to two isolates of *M. anisopliae* at concentration of 1x 10^7 conidiaml^{-1}



3.4 Effect to *P. xylostella* larval stage on susceptibility to *B. thuringiensis* and *M. anisopliae*

The different larval stages of DBM exposed to *B. thuringiensis* and *M. anisopliae* varied in their susceptibility to infection by the two pathogens. Earlier stage of 2nd - instar larvae was more susceptible than the older larval stages. The LD_{50} values were 7.1 x 10^4, 3.3 x 10^5 and 2.7 x 10^6 spores/ml for 2nd 3rd and 4th instars, respectively (Table 8). The 2nd - instar larvae therefore had the lowest LD_{50} value. Larvae that survived revealed signs of infection by swelling and turning yellowish in colour. They soon stopped feeding and exhibited disorientation behavior leading to early pupation on the upper lid of the Petri dish. The resultant pupae were malformed and a similar case of deformity was evident on those adults that emerged after treatment.

The LD_{50} values for second and third - instar larvae treated with *M. anisopliae* were 8.9 x 10^5 and 3.2 x10^6 conidia ml^{-1}, respectively (Table 9).

Table 8. Probit-mortality-dose regression parameters for three *P. xylostella* instars treated with *B. thuringiensis* isolate, Px-K3

Instar	LD_{50s} and 95% fiducial limits (Spores ml^{-1})	Slope ± SE
2nd	7.1×10^4 (5.2×10^4 - 9.1×10^4)	2.18 ± 0.25
3rd	3.3×10^5 (2.5×10^5- 4.4×10^5)	1.34 ± 0.12
4th	2.7×10^6 (2.7×10^6- 4.7×10^6)	1.38 ± 0.10

Table 9. Probit-mortality-dose regression parameters for two *P. xylostella* instars treated with *M. anisopliae* isolate, IC20.

Instar	LD_{50s} and 95% fiducial limits (conidia ml^{-1})	Slope ± SE
2nd	8.9×10^5 (5.1×10^5 - 13.5×10^5)	1.07 ± 0.12
3rd	3.2×10^6 (2.2×10^6 - 4.6×10^6)	0.94 ± 0.09

3.5 Prospects of *B. thuringiensis* and *M. anisopliae* in the control of *P. xylostella* in green house.

Efficacy of two biopesticides and a chemical pesticide was determined in the green house to control the DBM. The damage inflicted by the larvae to cabbage leaves varied with different treatments (Table 10). There was no significant difference in the level of damage in all treatments during the first two days after treatment ($P < 0.05$). But at eighth day post-inoculation, potted plants treated with *B. thuringiensis* isolate Px-K3 recorded the lowest leaf damage while the ones treated with synthetic chemical Karate scored the highest damage rate.

Larval mortality also varied with treatments (Table 11). Mortality in pots treated with *B. thuringiensis* isolates Px-K3 and Dipel 2x was observed two days after treatment. Isolate Px-k3 recorded the highest mortality of 96 % at eight days post treatment. Synthetic chemical Karate exhibited an initial knockdown effect by reducing *P. xylostella* larvae after the second day. However it recorded the lowest mortality compared to other treatments. (Figure 8). Larvae treated with Karate were weak at the initial stage following treatment but regained and continued causing damage to the cabbages. This is an indication that *P. xylostella* could have mounted resistance to the chemical. The observation is in accordance to the report given by farmers about the inefficacy of this chemical in controlling the diamond back moth in Kiambu area during the field collection of *P. xylostella* larvae.

Mortality of larvae treated with *M. anisopliae* was observed after the fourth day following inoculation. The performance of fungal isolate was slightly higher compared to

that of *B. thuringiensis* commercial product Dipel 2X®. However, there was no significance difference between Dipel 2X® and *M. anisopliae* at the eighth day ($P<0.05$).

Table 10. Cabbage leaf damage score rates due to DBM infestation

Treatment	Day 0	Day 2	Day 4	Day 6	Day 8
Control	1.0 ± 0.0a	2.0 ± 0.0a	3.6 ± 0.24a	4.8 ± 0.2a	5.0 ± 0.0a
Dipel 2X®	1.0 ± 0.0a	2.0 ± 0.0a	2.0 ± 0.0b	2.4 ± 0.24cd	2.8 ± 0.2c
IC20	1.2 ± 0.2a	2.0 ± 0.0a	2.4 ± 0.24b	2.8 ± 0.2c	3.0 ± 0.0c
Karate ®	1.0 ± 0.0a	2.0 ± 0.0a	3.6 ± 0.24a	4.2 ± 0.2b	4.2 ± 0.2b
Px-K3	1.0 ± 0.0a	2.0 ± 0.0a	2.0 ± 0.0b	2.0 ± 0.0d	2.2 ± 0.2d

Means in the same column with the same letter are not significantly different at $P< 0.05$ by Student-Newman-Kuels.

Table 11. Percentage mortalities of DBM larvae following spray application with biopesticide and chemical in potted cabbage plants.

Treatment	Day 0	Day 2	Day 4	Day 6	Day 8
Control	0	1.6 ± 1.2d	4.8 ± 1.0d	6.4± 0.7d	6.8 ± 1.0d
Dipel 2X®	0	60 ± 1.8b	72.4 ± 2.6b	74.8 ± 3.4b	78.8 ± 3.4b
IC20	0	9.8 ± 2.3c	59.2 ± 3.0c	74.8 ± 1.9b	80 ± 1.78b
Karate ®	0	56.0 ± 1.9b	58.8 ± 1.5c	60.4 ± 1.7c	62.4 ± 2.4c
Px-K3	0	73.2 ± 2.3a	90.8 ± 1.0a	95.6 ± 1.7a	96.4 ± 1.3a

Means in the same column with the same letter are not significantly different at $P< 0.05$ by Student-Newman-Kuels.

Figure 8. Damage induced on potted cabbages by *P. xylostella* in the greenhouse experiment

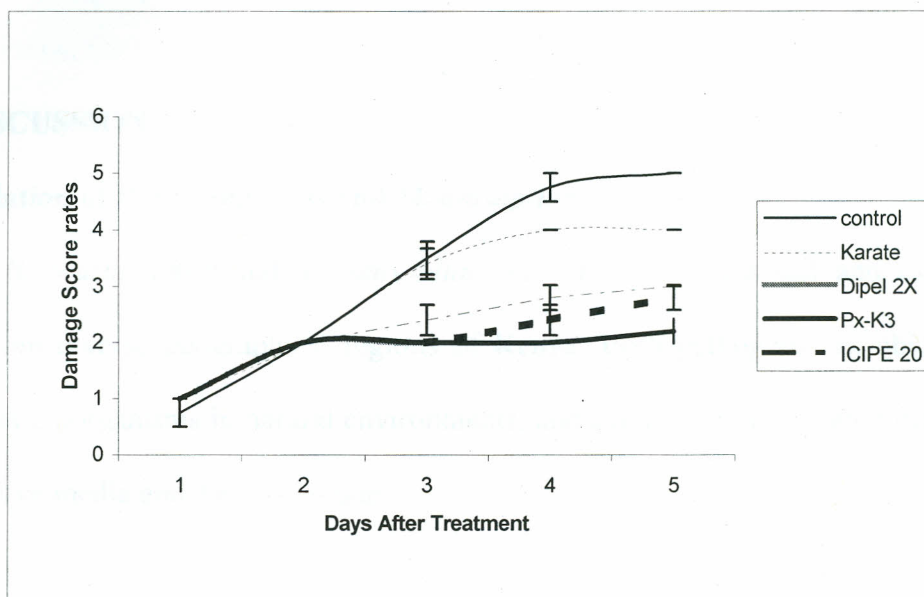
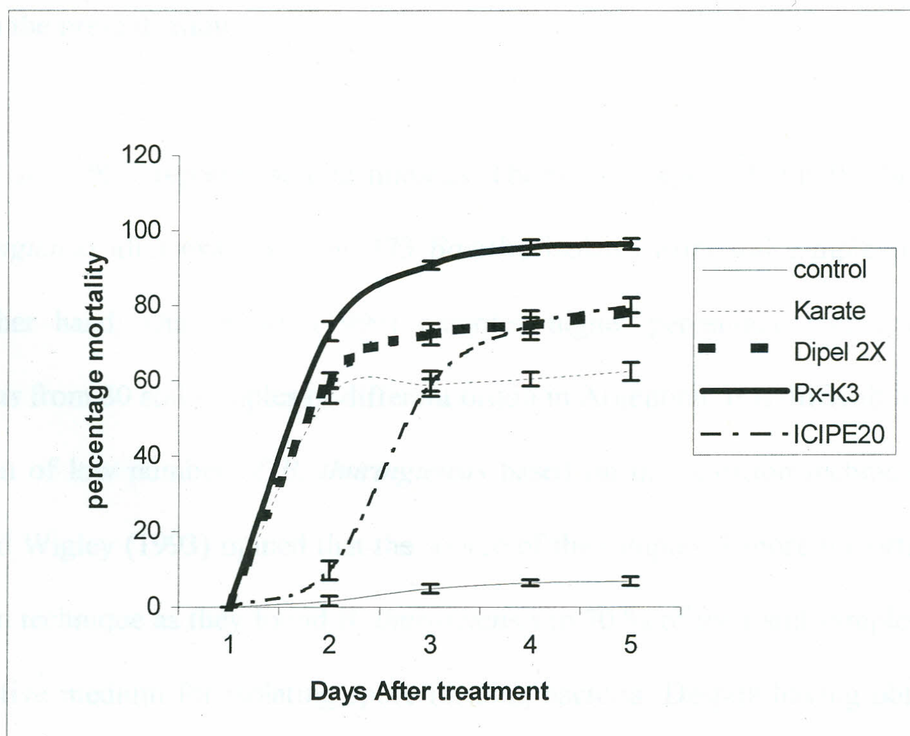


Figure 9. Performance of two biopesticides and a chemical pesticide on *P. xylostella* in the green house.



CHAPTER FOUR

4.0 DISCUSSION

4.1 Isolation of *B. thuringiensis* and *M. anisopliae*

Strains of *B. thuringiensis* and *M. anisopliae* were isolated from soil and cadavers obtained from diverse geographical regions of Kenya. Both pathogens are ubiquitous soil-borne microorganisms in natural environments, and can be readily isolated from soil using selective media and 'bait technique'.

Isolation of *B. thuringiensis* has been reported from soil (Martin and Travers, 1989) and phylloplane (Smith and Couche, 1991) since its initial isolation from a diseased silk moth, *Bombyx mori*. Low number of *B. thuringiensis* isolates was found from the samples collected in the present study.

DeLucca *et al.* (1981), reported similar findings. The authors reported only 0.5 % isolates of *B. thuringiensis* after examining 46,373 *Bacillus* isolates from soil samples in USA. On the other hand, Dias *et al.* (1999) recorded higher percentage recovery of *B. thuringiensis* from 80 soil samples of different origin in Argentina. It is difficult to justify the isolation of low number of *B. thuringiensis* based on the isolation technique only. Chilcott and Wigley (1993) opined that the source of the samples is more important than the isolation technique as they found *B. thuringiensis* in 70 % of their soil samples, using a non-selective medium for isolating spore-forming bacteria. Despite having obtained a

low number of *B. thuringiensis* isolates the technique suggested by the WHO (1985) was simple, fast and permitted the isolation of other *Bacillus* species. Use of culture media with antibiotics for subcultures only (Yousten, 1991) permitted rapid and accurate identification of strains of *B. thuringiensis*.

Three isolates of *M. anisopliae* were isolated from the soil samples in this study. Many workers have used a variety of fungicides and antibiotics in selective media to isolate *Metarhizium* species from soil (Zou *et al.,* 1993). This low number could be attributed to the isolation technique. Lomer (1995) has recommended the use of a wide range of media when isolating fungi from the natural substrata. Beitharz *et al.* (1982) found that media incorporating the fungicide dodine (N- dodecylguanidine monoacetae) successfully isolated *M. anisopliae* from garden soil while normal soil plating media did not. Other investigators have used the '*Galleria* bait method' (Zimmermann, 1986) for the detection of entomopathogenic fungi. Bidochka *et al.* (1999) isolated 357 isolates of *M. anisopliae* in 266 soil samples in Ontario, Canada using this technique, which involves baiting the soil with waxworm larvae, *Galleria mellonella.*

Isolation of insect pathogens is the first step in the development of pathogens as microbial control agents for insect pests. Therefore, the results of this study contribute towards this objective.

4.2 Strain selection

The results on the screening showed differences in the pathogenic activity of isolates of *B. thuringiensis* tested against *P. xylostella.* Several workers have reported differences in the pathogenicity of *B. thuringiensis* strains to Lepidoterans pest species (Brownbridge and Onyango, 1992; Dias *et al.*, 1999; Navon, 2000). There were also differences in the pathogenicity of isolates of *M. anisopliae* against *P. xylostella* larvae. The results are in accordance with published works on other insect pests. Ekesi *et al.* (1998) reported considerable variations in the pathogenicity of different strains of *M. anisopliae* against legume thrips *Megalulothrips sjostedi.* Moorhouse *et al.* (1993) observed differences in virulence of *Metarhizium* isolates from a range of different hosts and different locations on vine weevil, *Otiorhynchus sulcatus.*

It is generally admitted that the most virulent entomopathogens are those derived from the target insect species or closely related species from where they were isolated (Soares *et al.*, 1981; Poprawski *et al.*, 1985; Glare and Milner, 1991). This was true in case of B. *thuringiensis,* isolate Px-K3 isolated from dead *P. xylostella* larva. The most pathogenic isolate of *M. anisopliae,* ICIPE 20, was isolated from the soil. Maniania (1992) reported similar observation of the virulence of *M. anisopliae* strains isolated from the soil to larvae of *Busseola fusca* (Fuller) and *Chilo partellus* (Swinhoe).

Isolates of *B. thuringiensis* obtained from the ICIPE's germplasm were virtually non-toxic to *P. xylostella* larvae. Differences in insecticidal activity have been reported by many workers using purified delta - endotoxin preparations. They found differences in

insecticidal activity between the strains of the same subspecies (Jaquet *et al.,* 1987). Mohan and Gujar (2001) showed that the strains producing a mixture of delta- endotoxin crystal viz, *Bt kurstaki* HD-1 and *Bt aizawai* HD-137 were more toxic than strains producing a single type of delta - endotoxin like *Bt kurstaki* HD-73 and *Bt kenyae* HD-136. Dilawari *et al*. (1996) ascribed the superiority of *Bt kurstaki* HD-1 over *Bt kurstaki* HD-73 against *P. xylostella* to be as a result of composition in crystal proteins.

Although molecular characterization of the local isolates have yet to be determined, the variation observed in the study could be as a result of differences in the size and molecular arrangements of the proteins and the amino acids in the delta - endotoxin crystal. According to Jaquet *et al.* (1987), variability exists in the amino acid sequence lying within the active fragment and these differences may affect the make-up, and hence the potency of the different delta - endotoxins towards a particular pest species.

It was observed during bioassays that *M. anisopliae* strain ICIPE 20 had antifeedant activity on *P. xylostella* larvae. The cabbage leaf damage was significantly reduced on leaf discs treated with this isolate than other isolates of *M. anisopliae*. Some *M. anisopliae* isolates are known to secrete cyclic depsipeptides called dextrusins that have antifeedent properties (Roberts, 1981). Recently, Amri *et al*. (1999) reported antifeedant property of destruxin from *M. anisopliae* against *P. xylostella* and *Phaedon cochleariae* in cabbage. The mechanism in which isolate ICIPE 20 causes antifeedent effect to larvae of *P. xylostella* is not clear since dextrusin is produced in liquid media and during infection process. Further studies are therefore required to elucidate this mechanism.

The antifeedent property of isolate ICIPE 20 could be an advantage to compensate the slow speed of kill that characterize fungal biopesticides, and should be of practical importance in the management of *Plutella xylostella.*

In conclusion, four isolates of *B. thuringiensis* (MR1, KF-2, NA-2 and Px-K3) and two of *M. anisopliae* were highly pathogenic to larvae of DBM and had the lowest LT_{50} values, and may therefore be considered to have a greater potential for inclusion in the management programmes of *P. xylostella*

4.3 Dose- mortality responses

Mortality was dose dependent with both *B. thuringiensis* and *M. anisopliae*. High doses caused high mortality within short time than lower doses. Many workers using various pathogens and insect pests have reported the effects of dosage on mortality. It is importance to evaluate dose response in characterizing the host pathogen interaction. Low mortalities at low dose levels of *M. anisopliae* were probably related to reduced conidial attachment on the larval integument. Assessment of rigorous dose response showed that at least 1800 conidia of *B. bassiana* strain CS-1 should adhere to a single DBM larva to induce successful infection (Yoon *et al.*, 1999).

It is of great importance to determine the efficacy of various doses for the effective field application and economic use of *B. thuringiensis* and *M. anisopliae* in the control of *P. xylostella*. This would help to curtail the misuse of these biopesticides due to wastage by using high doses, which might not be economically sound for a resource poor farmer, and

also to avoid subjecting the pest to sub lethal doses, which could lead to development of resistance. The dose response relationship therefore provides an initial experimental base upon which further studies evaluating these biological systems can build.

4.4 **Susceptibity of different larval stages to *B. thuringiensis* and *M. anisopliae***

Both *B. thuringiensis* and *M. anisopliae* were more effective on first and second larvae instars than third and fourth instars larvae of *P. xylostella*. Several workers have reported similar findings. Kariuki (1987) observed that young larvae of *C. partellus* and *B. fusca* were more susceptible to *B. thuringiensis* than mature larvae. It has been shown in the laboratory and field bioassays that third instar larvae of Lepidopteran are less susceptible to *B. thuringiensis* products than the younger instar larvae (Navon *et al.*, 1990).

Studies by Ekesi *et al.* (1998) showed that larvae of *M. sjostedi* were less susceptible to infection by *M. anisopliae*. The presence of exuviae of *P. xylostella* larvae was observed on the leaf discs following infection with *M. anisoplae*. This is a good indication that the larvae have a mechanism of counteracting infection through casting of the cuticle. The differential susceptibility at two larval stages of DBM by *M. anisopliae* could be ascribed to interaction between the integument being penetrated by the fungus and the molting of the larval stages. It is known that insects may escape fungal infection by casting the infectious inoculum with their exoskeleton at the time of molting (Vey *et al.*, 1977).

The outcome of this differential larval susceptibility suggests that *B. thuringiensis* and *M. anisopliae* applications, with the aim of controlling *P. xylostella* should be synchronized

with the occurrence of early instars. Such synchrony would not only provide maximum susceptibilty, but it would also allow earlier production of the secondary inoculum (thus secondary infection) while minimizing the length of time that DBM larvae would be damaging the cabbage. Therefore, targeting the early instars of *P. xylostella* would enable farmers to realize less damage before economic injury is reached. This is of great importance for farmers who target the market for their produce where even the cosmetic damage could affect the market value of the produce.

4.5 Prospects of *B. thuringiensis* and *M. anisopliae* for the control of *P. xylostella* in green house

Copenhagen cabbage treated with local isolates of *B. thuringiensis* and *M. anisopliae* supported lower numbers of larvae than those in the control. Therefore the two strains were effective in suppressing the *P. xylostella* numbers in the green house. Shelton *et al.* (1998) observed significant reduction of DBM population and damage ratings when *B. bassiana* and *M. anisopliae* were tested against *P. xylostella* in screen house. Similarly, concentrations of 1 x 10^8 and 1 x10^9 spores of *M. anisopliae* and *Paecilomyces fumoroseus* exposed to cabbage root flies (*Delia radicum*) reduced the root damage of head cabbage by 20- 70% compared with untreated controls (Vanninen *et al.*, 1999)

Development of infection by the *M. anisopliae* strain, ICIPE 20 was slower and lower mortality was recorded in the green house than the pattern shown in the laboratory assays. This may be ascribed to unfavourable interaction of the pathogen and the green house environment. McCoy, (1981) reported that spore

germination of most entomopathogenic deuteromycetes requires relative humidity of above 90% and optimal temperatures of around 25°C. A correlation between optimum temperatures for fungal growth and fungal infection has also been reported by several workers (Maniania , 1992).
M. anisopliae strain IC20 however still showed considerable reduction in cabbage infestation in the green house despite the fluctuation in temperatures. *Plutella xylostella* populations generally do not live at constant temperatures in nature but are subject to diurnal and seasonal fluctuations. Therefore, from an ecological perspective *M. anisopliae* would have good potential for the management of DBM at different field conditions.

An important observation made was the toxicological effects of Karate® on *P. xylostella* larvae, where high damage was recorded on cabbage even after treatment. This is an indication that *P. xylostella* could have mounted resistance to the chemical. The observation is in accordance to the report given by farmers about the inefficacy of this chemical in controlling the diamondback moth in Kiambu area during the field collection of *P. xylostella* larvae.

Generally, the preliminary studies in the green house revealed that both local isolates of *B. thuringiensis*, Px-K3 and *M. anisopliae* strain, ICIPE 20 were still potentially able to suppress the diamondback moth larvae population significantly compared with the control and synthetic chemical, Karate®. Although the data obtained from this green house experiment do not suggest the same level of

performance of *B. thuringiensis* and *M. anisopliae* strains in the field, both should be qualified as candidates for thorough filed testing as biocontrol agents.

CHAPTER SIX

6.0 CONCLUSION

It has come out that a major contribution on cabbage production has been achieved from this research. The study has revealed that local isolates of *B. thuringiensis*, Px-K3 and *M. anisopliae,* ICIPE 20 have a great potential for the management of the diamondback moth and hence a good component of the integrated pest management strategies. Good control achieved in the laboratory and green house experiments is encouraging for field extrapolation of the results. Reduction in larval population could mean a reduction in the rate of population build up hence low damage. Rapid reduction in feeding at the larval stage show that the pest status of infected larvae can be markedly reduced by *B. thuringiensis* and *M. anisopliae* even though mortality is not immediate.

Increasing regulatory constraints and the cost of registration coupled with insecticides makes the utilization of biopesticides more appealing today than in the recent past.

By developing and commercializing biopesticides, the risk of misuse of synthetic pesticides among farmers who generally have inadequate knowledge of pesticide safety and insufficient means to purchase protective equipment is reduced. The role of these biopesticides may as yet not have a clear impression of a pest reducing factor in the mind of a farmer, probably because entomopathogens are slower acting mortality agents. However biopesticides have a market potential in East Africa for the control of lepidopteran pests, especially in vegetables where stringent measures on residual limits are set. Population and economic trends, farming practices and technological

developments in agricultural sector will however determine the demand for these entomopathogens.

6.1 RECOMMENDATIONS

A thorough survey on the natural occurrence of *B. thuringiensis* and *M. anisopliae* should be carried out in all high potential agro-ecological zones to supplement this study. Molecular characterization to ascertain difference in pathogenicity of the local isolates of *B thuringiensis* and *M. anisopliae* is also essential. Further studies are required to elucidate the mechanism in which isolate ICIPE 20 causes antifeedent effect to larvae of *P. xylostella.* Field tests should be carried out at different growing seasons and geographical areas to establish the field efficacy of these entomopathogens. The combination of *B. thuringiensis* and *M. anisopliae* has not been evaluated in detail but, in view of the high efficacy of *M. anisopliae* on sucking insects, such a combination in suitable formulation might provide a useful strategy in combating the adult moth. Training of users is a crucial aspect in adoption of any technology. Therefore, farmers and extension staff should be trained in production and utilization of entomopathogenic *B. thuringiensis* and *M. anisopliae.*

APPENDIX 1. Isolates of *B. thuringiensis* and *M. anisopliae* isolated from field samples

***B. thuringiensis* isolates**	**Date isolated**	**Area of isolation**	**Description of the site.**
KZ - 3	22 - 08 - 2000	Kibwezi	Under the trees
SH - 4	22 - 08 - 2000	Shimba hills	In grassland
MR - 1	22 - 08 - 2000	Meru	Bareground
NA - 3	28 - 08 - 2000	Naivasha	Under acacia trees
NA - 2	28 - 08 - 2000	Naivasha	In cabbage farm
ML - 4	28 - 08 - 2000	Molo	In grassland
KF - 2	01 - 09 - 2000	Kilifi	In KARI farm
SH - 4	01 - 09 - 2000	Shimba hills	In grassland
MP- 3	06 - 09 - 2000	Mbita point	Under trees
SK -5	06 - 09 - 2000	Suneka	Elevated ground
NY -2	10 - 09 - 2000	Nyeri	In cabbage farm
Px- K3	07 - 10 - 2000	Kijabe	In cabbage farm

APPENDIX 2. Dead & diseased larvae collected during the field Survey

REGION	DATE OLLECTED	NUMBER OF DBM	HOST PLANT
Wundanyi	14- 06- 2000	2	Cabbage
Voi	18 -06 -2000	8	Cabbage
Nyeri	12 - 07 - 200	6	Cabbage
Nakuru(Bahati)	20- 07- 2000	7	Kales
Nyangori (Vihiga)	22- 07- 2000	6	Kales
Mbita point	22- 07-2000	4	Cabbage
Kiambu (Kijabe)	07- 05- 2001	3	Kales

APPENDEX 3: Isolates obtained from ICIPE Culture collection

Metarhizium anisopliae	***Bacillus thuringiensis* isolates**
IC 18	Bt252/2
IC20	Bt 3
IC30	HD 220
IC40	M37
IC60	
IC63	
IC69	
IC75	
IC78	

REFERENCES:

Abbott W.S. (1925). A method for computing the effectiveness of an insecticide. *Journal of economic entomology* **18**: 265-267

Abdul R.F and Ellar D.J. (1999). Toxicity and receptor binding properties of *Bacillus thuringiensis* Cry 1C toxin active against both Lepidoptera and Diptera. *Journal of Invertebrate Pathology* **73**(1): 52-58

Amri B., Ibrahim, L., and Butt, T.M. (1999). Antifeedant properties of Destruxins and their potential use with the Entomogenous fungus *Metarhizium anisopliae* for improved control of Crucifer pests. *Biocontrol Science ad Technology,* **9:** 487-498.

Beitharz V.C., Parbery, D.G., and Swart, H.J. (1982). Dodine: A selective agent for certain soil fungi. *Trans. Br. Mycol. Soc.* **79:** 507-511.

Bidochka M.J., Kasperski, J.E., and Wild G.A.M. (1999). Occurrence of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-Northern habitats. *Canadian journal of Biotechology,* **76**: 1198-1204.

Boucias D.G., Schobury, E.L., and Allen, G.E. (1982). The relative susceptibility of six noctuid species of infection by *Nomuraea rileyi* isolated from *Anticarsia gemmatalis. Journal of Invertebrate Pathology* **39:** 238-240.

Brownbridge M., and Onyango, F. (1992). Screening of exotic and locally isolated *Bacillus thuringiensis* (Berliner) strains in Kenya for toxicity to the spotted stem borer, *Chilo partellus* (Swinhoe). *Tropical pest management,* **38:**77-81.

Burgerjon A. (1956). Pulverisation de poudrage au laboratoire pardes preparations pathogenes insecticides. *Annals of Epiphytology* **4:** 677-688.

Butt T.M. (1990). Fungal infection process: A minireview In: *Proceedings and Abstracts 5th Internatioanal Colloquim on Invertebrate Pathology and microbial control.* Adelaide, Australia, pp 121-124.

Chandler D., Hay, D. and Reid, A.P. (1997). Sampling and occurrence of entomopathogenic fungi and nematodes in U.K soils. *Applied Soil Ecology* **5,** 133-141

Charnley A.K., Samuels, R.A., Renolds S.E. (1988). Calcium channel activation of insect muscle by destruxins, insecticidal compounds produced by entomopathogenic

fungus, *Metarhizium anisopliae*. *Comparative Biochemical Physioliology*. **90:** 403 - 412.

Chelliah S., Srinivasan, K. (1986). Bio- ecology and management of diamondback moth in India. *Journal of Applied Entomology*. **98**: 249-55.

Crickmore N., Zeigler, D.R., Feitelson, J., Schnept, E., VanRie, J., Lereclus, D., Baum, J., and Dean D.H. (1998) "*Bacillus thuringiensis* toxin nomenclature" *Microbiology and Molecular Biology Review*, **62:** 807-813

de Barjac H. and Frachon, E. (1990). Classification of *Bacillus thuringiensis* strains. *Entomophaga* **35:** 233-240

Delucca A.J., Simpson J.G., and Larson, A.D. (1981). *Bacillus thuringiensis* distribution in soils of the United States. *Canadian Society of Microbiology*. **27**: 865-870

Dias S.C., Sagardoy, M.A, Silva, S.F., and Dias, J.M.C.S. (1999). Characterization ad pathogenic evaluation of *Bacillus thuringiensis* and *Bacillus sphaericus* isolates from Argentinean soils. *Biocontrol*, **44:** 59-71

Ekesi S., Maniania, N.K., Onu, I., Lohr, B. (1998). Pathogenicity of entomopathogenic fungi (Hyphomycetes) to the legume flower thrips, (Trybom) (Thysan., Thripadae). *J. Appl. Ent*. **122:** 629-634 *Megalurothrips sjostedi*

Fast, P. G., (1981). 'The crystal toxin of *Bacillus thuringiensis'*. In microbial control of pests and plant diseases 1970-1980 (ED. H.D Burges). pp 221-248, Academic press London.

Ferron P. (1978). Biological control of insect pests by entomogenous fungi. *Annual Review of Entomology*. **23:** 409-422.

Furlong M.J and Pell, J.K. (1996). Interactions between the Fungal Entomopathogen *Zoophthora radicans* and Brefeld (Entomophthales) and Two Hymonepteran Parasitoids Attacking the Diamondback moth, *Plutella xylostella* L, *Journal of invertebrate Pathology*, **68**: 16-21

Furlong M.J., Pell, J.K., Ong, P.C and Syed, A.R. (1995). Field and laboratory evaluation of a sex pheromon trap for the autodissemination of the fungal entomopathogen *Zoopthora radicans* by the diamondback moth, *Plutella xylostella*. *Bulletin of Entomological Research*. **85** (3): 331-337.

Gabriel B.P. (1968). Histochemical studies of the insect cuticle infected by the fungus *Entomophthora coronata*. *Journal of Invertebrate Pathology*. **11**:82-89

Gill S.S., Cowles E.A. and Pietrantonio P.V. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Annual Review of Entomology* **39**: 615-636.

Gillespie A.T. Claydonn, N. (1989). The use of entomopathogenous fungi for pest control and the role of toxins in pathogenesis. *Pesticide Science.* **27:** 203-15.

Glare T.R. and Milner, R.J. (1991). Ecology of Entomopathogenic Fungi. In Handbook of Applied Mycology, vol.2: Humans, Animals and insects (eds.D.K. Anra, L. Ajello and K.G. Mukerji) pp 547-612. Marcel Dekker inc., New york.

Haber H.E., Luthy, P. Elbresold, H. R., and Cordier, J. R. (1981). The subunits of the parasporalcrystal of *Bacillus thuringiensis*: size, linkage and toxicity. *Arch. Microbial.* **129**:14-18.

Hajek A.E, and Leger R.J. (1994). Interaction between fungal pathogens ad insect hosts. *Annual Review of Entomology.* **39**: 293-322.

Hall R.A., Papierok, B. (1982). Fungi as biological control agents of arthropods of agricultural and medical importance. *Parasitology* **84**: 205- 240

Harcourt D.G. (1960). The biology and ecology of the diamondback moth, *Plutella maculipennis,*(Curtis) (Lepidoptera: Plutellidae), in Eastern Ontario. Natural enemies. *The canadian Entomologist 92:* 419-428

Hassan A.E.M., Dillion, R.J. and Charnley, A.I.L. (1989). Influence of accelerated germination of conidia on the pathogenicity of conidia of *Metarhizium* for *Manduca sexta. Journal of Invertebrate Patholology* **54**: 277-279.

Heimpel, A. M. Angus, T.A. (1959). The site of action of crystalliferous bacteria in Lepidoptera larvae. *Journal of Insect Pathology.* **1**. 152-170.

Hoffmann M.P and Frodsham A.C. (1993). Natural enemies of vegetable insects pests cooperative Extension, Cornell University, Ithaca, NY. Pp 63

Hofmann C., Vanderbruggen, H., Hofte, H., VanRie, J., Jansens, S., and Van Mellaert, H., (1988). Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high affinity binding sites in the brush border membrane of target insect midguts. *Proceedings of Natural Academy of Science*. USA. **85,** 7844-7848.

Hofte H. and Whitely H.R. (1989). Insecticidal crystal protein of *Bacillus thuringiensis. Microbiology Review* **53**:249-55.

Humber R.A. (1992). Collection of entomopathogenic fungal cultures: Catalogue of strains. U.S. Department of Agriculture, Ithaca, NY. Publication ARS-110.

ILACO, (1981). Agircultural Compendium for Rural Development in the Tropic and subtropics, pp 234- 54. Elsevier scientific publishing company, Amsterdam.

Jaquet F, Hutter, R and Luthy P. (1987). Specificity of *Bacillus thuringiensis* delta-endotoxin. *Applied and Environmental microbiology,* **53**, 500-504.

Kariuki C.W, (1987) " The Laboratory and field efficacy of *Bacillus thuringiensis* (Berliner) Against Tropical cereal stem borers (*Chilo partellus*(Swinhoe) and *Busseola fusca* (Fuller) and legume pod borer (*Maruca testulalis).* M.Sc. Thesis, University of Nairobi, Kenya.

Kfir, R. (1998). Origin of the diamondback moth. (Lepidoptera: Plutellidae). *Entomological society of America.* **91**: 164-167

Knowles B.H (1994). Mechanisms of action of *Bacillus thuringiensis* insecticidal delta-endotoxins. *Advanced Insect Physiolology.* **24:** 275-308.

Kooyman C. and Abdalla, O. M. (1998) Application of *Metarhizium flaroviride* (Deuteromycotina: Hyphomycetes) spores against the tree locust, *Anacridium melonorhodon* (Orthoptera: Acrididae) in Sudan. *Biocontrol Science and Technology:* **8**, 215-219.

Lampkin N.H. and Padel, S. (eds). (1994). The Economics of organic Farming: *An international perspective.* CAB International, Wallingford. UK. pp 486.

Le Van Trinh (1996). Diamond back moth in the North of Vietnam and proposal of control program. In *The management of diamond back moth and other crucifer pests: Proceedings of the third international Workshop,* Kuala Lumpur, Malaysia. Malaysia Agricultural Research and development institute, pp 47-54.

Lisanky S. G., Quinlam, R. J., and Tassoni, G. (1993). The *Bacillus thuringiensis* production Handbook. CPL Scientific Press, United Kingdom, pp.12

Lomer H.C, (1995). Identification of some common entomopathogenic fungi. *Lubilosa technical Bulletin,* **2**: 9-11.

Lu Z.Q., Chen, L.F., and Zhu, S.D. (1988). Studies on the effect of temperature on the development, fecundity and multiplication of *Plutella xylostella* L. *Insect Knowl.* **25**: 147-49.

Luthy P., Cordier, J.L. and Fischer, H. M. (1993). *Bacillus thuringiensis* as a bacterial insecticide: basic consideration and application. In microbial and viral pesticides (Ed. E. Kurstak), pp 35-74. Marcel deker, New York.

Madumadu G.G., Sikinyi, E.O., Mwamba D.K. (1991). Vegetables for domestic and export markets. *Proceedings of Review workshop on National Horticultural Research Programme* Thika, Kenya pp. 41-63.

Magaro J.J., and Edelson, J.V. (1990). Diamondback moth (Lepidoptera: Plutellidae) in south Texas: a technique for resistance monitoring in the field. *Journal of Economic Entomology*. **83,** 1201-1206.

MALDM (1998). Crop production statistics (Ministry of Agriculture, Livestock Development and Marketing). *Annual Report* 1998 Kenya.

Maniania N.K. (1992). Pathogenicity of entompathogenic fungi (Hyphomycetes) to larvae of the stemborer, *Chilo partellus* (Swinhoe) and *Busseola fusca* (Fuller). *Insect Science Application.* **13**, 691-696.

Martin P.A.W. and Traver, R.S. (1989). A worldwide abundance and distribution of *Baccilus thuringiensis* isolates. *Applied Environmental Microbiology.* **55**:1101-1104.

McCoy C.W. (1981). Pest control by the fungus *Hirsutella thompsonii.* In: Microbial control of Pests and Plant Diseases 1970-1980 (Ed: Burges, H.D). Academic Press, New York. pp.449-512

Mohan M, Gujar, G.T. (2001). Toxicity of Bacillus *thuringiensis* strains and commercial formulations to the Diamondback moth, *P. Xylostella* (L). *Crop protection,* **20**: 311-316.

Moorhouse E.R., Gillieupie, A.T., and Charnley, A.K. (1993). Laboratory selection of *Metarhizium* isolates for control of vine-weevil larvae (*Otiorhynchus sulcatus*). *Journal of Invertebrate Pathology.* **62:** 15.21

Navon A, Klein, M, and Braun, S. (1990). *Bacillus thuringiensis* potency bioassay against *Heliothis armigera, Ecrias insulana*, and *Spodoptera littoralis* larvae based on standardized diets. *Journal of Invertebrate Patholology* **55:** 387- 393

Navon A. (2000). *Bacillus thuringiensis* insecticides in crop protection - Reality ad prospects. *Crop protection* 19: 270-275

Odour G.I., Lohr, B. and Seif A.A. (1996). Seasonality of major cabbage pests and incidence of their natural enemies in central Kenya; *Proceedings of Third international workshop*, Kuala,Lumpur, Malysia.

Ogol C.K.P.O and Spence J.R. (1997). Abundance, population dynamics and impact of the Luceana psyllid *Heteropsylla cubana* Crawford in maize –luceana agroforestry system in Kenya. In: thesis studies on the abundance, impact and natural enemies of the insect pests of okra and chillies and efficacy of neem products in their control. Muchemi, S. Kenyatta University.

Osir E.O., and Vundla, W.R.M. (1997). Characterization of the δ- endotoxin of a *B. thuringiensis* isolate active against tse tse, *Glossina mortisans*, and stem borer, *C. partellus. Biocontrol Science and Technology* **9**: 247-258.

Osterman H. and Dreyer, M. (1995). Vegetables and grain legumes, In the neem tree: Source of unique natural products for integrated pest management, medicine, industry and other purposes (Ed. H. Schmutterer). VCH publishers Inc. New York. pp. 392-403.

Pinley E. C. G (1975). Moths of southern Africa.Tafelberg publishers Ltd.

Poprawski T.J., Marchal, M., and Robert, P.H. (1985). Comparative susceptibility of *Otiorhynchus sulcatus* and *Sitona lineatus* (Coleoptera: Curculionidae) early stages to five entomopathogenic hyphomycetes. *Journal of Environmental Entomology.* **14**: 247-253.

Prior C. Jollands, P. Potourel, G. (1988). Infectivity of oil and water formulations of *Beauveria bassiana* (Deuteromycotina:Hyphomycetes) to the cocao weevil pest. *Biocontrol Science and Technology*. **2:** 312-316

Roberts D. W. (1981). Toxins of Entomopathogenic fungi. In: *Microbial control of pests and plant Diseases* (Burges. HD Ed.) Academic press, New York. pp. 441-464.

Salinas P.J. (1984). Studies on the behaviour of the larvae of *Plutella xylostella* (L) (Lepidoptera: Plutellidae), a world pest of cruciferous crops: Normal and "Spacing" behaviour. Turralba 34:77-84.

Shelton A.M Robertson, J.L Tang J.D., Perez, C. and Eigenbrode, S.D. (1993). Resistance of Diamondback moth to *Bacillus thuringiensis* subspecies in the field. *Journal of Economic Entomology* **39**: 47-79

Shelton A.M., Verdenberg, J.D., Ramos, M. and Wilsey W.T. (1998). Efficacy and persistance of *Beauveria bassiana* and other fungi for control of diamondback moth (Lepidoptera: Plutellidae) on cabbage seedlings. *Journal of entomological science*. **33**:142-151

Smith R.A. and Couche, G.A. (1991). The phyllophane as a source of *Bacillus thuringiensis* variants. *Applied Environmental Microbiology*. **57**: 311-315

Soares G.G., Marchal, M., and Ferron, P. (1981). Susceptibility of *Otiorhynchs sulcatus* (Coleoptera, curculionidae) larvae of *Metarhizium anisopliae* and *Metarhizium flavoviride* (Deuteromycotine, Hyphomycetes) at two different temperatures. *Journal of Environmental Entomology* **12:** 1886-1890.

Syed A.R. (1992). Insecticide resistance in diamondback moth in Malaysia. *Proceedings of the second international workshop in diamondback moth*, Shanhua, Taiwan 1992, pp. 437- 42

Tabashnik B.E. (1994). Evolution of resistance to *Bacillus thuringiensis*. *Annual review of Entomology* **39**:47-79

Talekar N.S. and Griggs, T.D. (1986). Diamondback moth management, *Proceedings of the First international workshop*, Tainan, Taiwan, pp 471. AVRDC.

Talekar N.S. and Shelton, A.M. (1993). Biology, Ecology and management of the diamondback moth. *Annual Review of Entomology*.**38**: 275-301.

Tindall H. (1983). Vegetables in the tropics, pp 90-145. Oxford University Press. London.

UNEP. (1995). Integrated pest management in the tropics: Current status and future prospects. John Wiley and Sons Ltd, Chichester.

Vandenberg J.D., and Ramos (1997). Screening of fungal isolates against larvae of the diamondback moth, *Arthropod Management*. Vol.**27** (in press).

Vanninen L., Hokkanen, H., Tyni- Justin, J. (1999). Attempts to control cabbage root flies, Delia radicum L. and Delia floralis (Fall.) (Dipt., Anthomyiidae) with entomopathogenic fungi: Laboratory and green house tests. *Journal of Applied Entomology*. **123**: 2, 107-113

Veeragavanatham D., Jawaharlal, M. and Ramadas, S. (1998). A guide on vegetable culture, pp 5-12. Suri association, Coimbatore, India

Vey A, and Fargues, J, (1977). Histological and ultra structural studies of *Beauveria bassiana* infection in *Leptinotarsa decemlineta* larvae during ecdysis. *Journal of Invertebrate Pathology* **30**: 207-215.

Wabule M.N. and Ikitoo, E.C. (1997). Research priorities and pest management concerns in export vegetable in Kenya. In: Towards improved pest management in export vegetables of Kenya. Report of a consultation meeting on needs and opportunities for improved pest management in export vegetables in Kenya. 26-27 February 1997, ICIPE, Nairobi, Kenya.

Wright S. P., Butt, T.M., Galainil-Wraight, S., Allee, L.L., Soper, R.S. and Roberts, D.W. (1990). Germination and infection process of the Entomophthoralean fungus *Erynia radican* on the potato leafhopper, *Empoasca fabae*. *Journal of Invertebrate Pathology* 56: 157-174.

Yoon C.S., Sung, G.H., Park, S.G., Lee and Lee J.O. (1999). Potential of the entomopathogenic fungi, *Beauveria bassiana* strain CS-1 as a biological control agent of *Plutella xylostella* (Lep., Yponomeutidae). *Journal of Applied Entomology*. **123:** 423-425.

Zimmermann G. (1986). Insect pathogenic fungi as pest control agents. *In: Biological Plant and health protection.* (FRANZ, H.G Ed.), Berlin: Fischer Verlag, Fortschrite der Zoologie Bd **32**, 231.

Zimmermann G. 1(993). The entomopathgenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. *Pesticide Science* **37**: 375-379

Zou Y. L., Milner, R.J., McRAE, C.F., and Lutton, G.G. (1993). The use of Dodine in selective media for the isolation of *Metarhizium* species from soil. *Journal of Invertebrate Pathology* **62**: 248-251.

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